ASSOCIATION OF *CULICOIDES MISSISSIPPIENSIS* HOFFMAN WITH *ILEX VOMITORIA* AITON, THE YAUPON HOLLY

By

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This work is dedicated to the memory of my mother, Margaret K. Stewart, whose warmth, encouragement, simplicity and unwavering love are the cornerstones of my life.

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Abstract of Dissertation Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

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Chairperson: D. L. Kline
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The association between the salt marsh biting midge, Culicoides mississippiensis Hoffman, and flowering male yaupon holly, Ilex vomitoria Aiton, was investigated in field and laboratory experiments in 1995 and 1996. Over 200 insect and floral volatile samples collected in spring 1995 were analyzed to identify phenological patterns of insect visitation in relationship to floral essential oil emissions. Insects were collected during one minute intervals, four times a day by AFS sweeper. Samples consisted mainly of thrips species (59.5%) and two ceratopogonids, Dasyhelea mutabilis Kieffer (30.6%) and C. mississippiensis (3.4%).

Adult C. mississippiensis were shown by anthrone tests to feed on nectar of male I. vomitoria flowers. Each of the three main insects visited flowers most frequently in the late

afternoon. Ceratopogonid visits were most abundant in the first half of the flowering season and during peak flowering. Thrips visits were most abundant in the second half of the flowering season during early and late flowering.

Twenty essential oils were identified from volatile samples of I. vomitoria by GC/MS. Two different patterns of emission were detected. One pattern was characterized by increasing output from early morning to mid-afternoon, followed by a decrease towards sunset. The second pattern showed increased output throughout the day up to sunset. Essential oils most strongly correlated with high sample numbers of C. mississippiensis were dimethyl benzene, α-pinene, β-pinene, 1,4-dimethyl benzene, 4,8-dimethyl-1,3,7-nonatriene, ethyl benzaldehyde, and ethenyl benzaldehyde. Those most strongly correlated with high sample numbers of D. mutabilis were dimethyl benzene, α-pinene, 1,4-dimethyl benzene, ethyl benzaldehyde, linalool oxide, 1-(2,4-dimethyl phenyl) ethanone, and phenyl acetonitrile. Future studies should examine attraction of C. mississippiensis to a blend in which those essential oils not highly correlated to capture of D. mutabilis are manipulated.

CHAPTER 1
INTRODUCTION

Culicoides mississippiensis Hoffman, the salt marsh biting midge, is a major pest along the Florida Gulf Coast. Breeding and foraging zones of C. mississippiensis cover huge tracts of coast land, overlapping with areas of human habitation and outdoor activities. Even at lower than peak population levels, biting rates frequently exceed several hundred per hour and can surpass two thousand per hour at peak levels (Lillie et al. 1988). The resulting interaction between midges and humans has negatively impacted local economies, recreational activities and public health.

No control program is currently being administered in Florida. Insecticidal mists and fogging provide only short term population reduction because of repopulation from untreated areas and development of resistance (Blanton and Wirth 1979). Moreover, salt marshes are considered ecologically sensitive areas and broadcast spraying of

pesticides is no longer an acceptable option. Impoundment, the flooding of breeding zones by construction of dikes with tidal gates, was implemented in the 1930s and 1940s with some success, but was stopped in the 1950s because of environmental concerns (Blanton and Wirth 1979).

Residents of severely infested localities such as Yankeetown, Levy County, Florida have mixed feelings about the need for control. During peak season, when C. mississippiensis is at extremely high densities in early morning and late afternoon, yard work is done in the middle of the day. While some people feel that control of their sand gnats would help improve property values, others fear the attraction their region would gain to developers and tourists (personal communications).

Nevertheless, reduction of Culicoides spp. populations should be a public health priority in part because of allergic reaction to bites, but more importantly because of the potential disease vectoring. Worldwide, Culicoides spp. are known vectors for many viral and filarial diseases (Linley et al. 1983). In the Caribbean, C. furens (Poey) transmits Mansonella ozzardi Manson, causative agent of mansonellosis, a filarial disease. Interaction between active C. furens

populations, found on both of Florida's coasts, and infected immigrants might result in establishment of mansonellosis in Florida. Bluetongue virus, a pathogen of livestock, is vectored by C. variipennis (Coquillett) in the United States and the eastern equine encephalitis virus has been isolated from pooled Culicoides in Georgia (Karstad et al. 1957). Little attention has been given to the vector potential of C. mississippiensis. An ecologically acceptable control program would not only bring relief in recreational and economic terms, but would serve to reduce the potential medical and veterinary impact of this pest as well as provide a model for control of other important Culicoides spp.

Culicoides mississippiensis has been reported along most of the U. S. Gulf Coast (Blanton and Wirth 1979). Culicoides mississippiensis was first described by Hoffman (1926) and female cotypes from Pass Christian, Mississippi deposited in the U. S. National Museum. Its appearance and biology are similar to those of C. hollensis (Melander and Brues), and until the late 1960s was thought to coexist with C. hollensis throughout its Gulf Coast range. Previous C. hollensis identifications for the Gulf Coast have been reclassified as C. mississippiensis (Blanton and Wirth 1979).

Extensive studies on the biology of C. mississippiensis have been carried out in the salt marshes of Yankeetown, Levy County, Florida. Culicoides mississippiensis has two annual population peaks, one in the spring and the other in the fall (Kline 1986). Emergent males and females feed on flower nectar. After their first oviposition event females convert to a haematophagous feeding behavior, showing preference for mammals as blood sources (Blanton and Wirth 1979). Host searching is highest at dawn and dusk but may occur at reduced levels throughout the daytime (Lillie et al. 1988). Oviposition occurs in salt marsh mud, mainly in stands of cordgrass, Spartina alterniflora Loisel (Kline and Wood 1988, Kline 1989), which is alternately covered by salt water or exposed to the air due to tidal patterns.

Oviposition sites are in proximity to scattered islets of land called hammocks. Hammock vegetation is characterized by white cedar, southern pine, live oak, cabbage palm, saw palmetto and holly. Yaupon holly, Ilex vomitoria Aiton, is the most common holly species in Yankeetown. It blooms from mid-March to mid-April when C. mississippiensis populations are at their highest.

Culicoides mississippiensis has been shown to be associated with flowering I. vomitoria (Lillie and Kline 1986). Field tests of attraction to detached flowers of I. vomitoria and seven other plants were carried out using a portable plexiglass olfactometer by the author in April 1993. In the absence of visual cues, significant attraction was demonstrated for both C. mississippiensis and Dasyhelea mutabilis Kieffer, a common gnat, only to flowers of I. vomitoria (unpublished personal data).

A follow-up study of C. mississippiensis visitation to flowering I. vomitoria and two other flowering plants, the saw palmetto Serenoa repens (Bartram) Small and the cabbage palm Sabal palmetto (Walter), was carried out in spring 1994. As flowering of I. vomitoria ended in mid-April that of S. repens began. Similarly, the flowering season of S. palmetto started in late May as that of S. repens was ending. Each of those plants is the dominant flowering plant in Yankeetown hammocks during its own flowering season. Visitations of C. mississippiensis to I. vomitoria were consistently high, whereas they were low to S. repens and non-existent to S. palmetto. Attraction to flowering I. vomitoria was shown to be a fact, but the nature of the attractant system remained

unknown. An examination of the association between C. mississippiensis and I. vomitoria is needed to identify possible candidate floral attractants.

The main research objective of the present study was to investigate the association of C. mississippiensis with I. vomitoria. Specific objectives of the study were 1) to characterize the floral phenology of an I. vomitoria population in a salt marsh hammock of Yankeetown, Levy County, Florida; 2) identify visitation patterns of the arthropod community associated with flowering male I. vomitoria to aid assessment of possible control on non-target arthropods; 3) establish the basis of primary attractancy of C. mississippiensis to flowering male I. vomitoria; and 4) identify the phenological patterns of chemicals emitted by flowers of male I. vomitoria during the entire flowering season and determine correlation between specific chemicals and high C. mississippiensis visitations.

CHAPTER 2
ILEX VOMITORIA AITON

Introduction

Ilex vomitoria Aiton, classified in the angiosperm family Aquifoliaceae, is a dioecious shrub or small tree growing to eight meters, found throughout the southeastern region of the United States (Figure 2-1a)(Hudson 1979). In Florida its distribution is coastal north of Brevard and Sarasota counties, inland in Polk, Marion, Alachua and Baker counties, and in all Panhandle counties except Leon and Calhoun (Figure 2-1b) (Wunderlin and Poppleton 1977).

The Aquifoliaceae are distinguished from other angiosperms by being oligostemonous (i.e., having the same number or fewer stamens as perianth members), syncarpous (i.e., having fused carpels) and by bearing fruit with few seeds and only one per carpel (Stebbins 1951). Family genera are divided into two groups, those that are deciduous and those that are evergreen, including Ilex (Wunderlin and Poppleton 1977). Ilex vomitoria differs from other species in

A

B

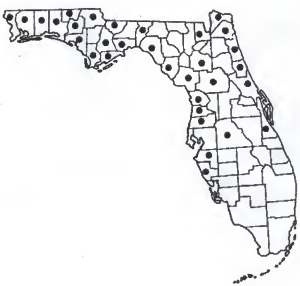
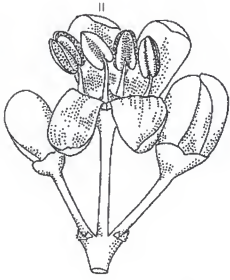


Figure 2-1. Distribution of Ilex vomitoria. A. United States (reprinted with permission of C. M. Hudson). B. Florida (reprinted with permission of R. P. Wunderlin and J. E. Poppleton.)

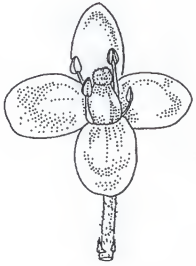
the genus in having completely crenate leaves (Figure 2-2a). Hume (1953) grouped American hollies according to persistence of leaves and fruit color. Species may be deciduous with black fruit, deciduous with red fruit, evergreen with black fruit, or evergreen with red fruit. Ilex vomitoria falls into the last category. It is also the only fasciculate holly native to the U. S. (Hume 1953), producing flowers on the previous season's stem growth.

In the Aquifoliaceae, nectar is produced at the base of petals or occasionally on the upper surface near the middle of petals (Loesener 1942). The flowers of I. vomitoria are tetramerous, with male flowers having four stamens and a rudimentary ovary while female flowers have four non-functional stamens and a pistil ending in a four-lobed stigma (Figure 2-2b,c). Bawa and Opler (1975) found that flowers of most dioecious plants in a lowland tropical semideciduous forest of Costa Rica are small and white to yellow or green. Male I. vomitoria flowers are small, cream-colored and more numerous than female flowers (Hu 1979). Flowers are borne in fascicles. Fascicles of male plants have several secondary flower clusters, called cymes, with up to five flowers per

A



B



C

Figure 2-2. A. Leaf of Ilex vomitoria. B. Male flower of I. vomitoria. C. Female flower of I. vomitoria. (reprinted with permission of C. M. Hudson.)

cyme while those of female plants have one reduced cyme with a maximum of seven flowers (Hu 1979).

Although I. vomitoria is related in geological time to Asian holly species, more recent lineage can be traced to an original stock in the Ouachita Mountains of western Arkansas and eastern Oklahoma (Hu 1979). It has thrived in the post oak savannah of east central Texas, an area intensively exploited for cattle grazing. Because its dense growth interferes with grazing and movement of equipment, I. vomitoria is controlled by herbicidal applications (Bovey et al. 1972, Duncan and Scifres 1983, Meyer and Bovey 1985). Adaptation to the drier conditions of seashore and hammocks has enabled I. vomitoria to establish itself in coastal areas (Hu 1979). The salt marshes invaded by I. vomitoria are protected wetlands and it is unlikely that the plants' growth will be controlled there as has been the case in Texas.

Historically, I. vomitoria has been important in both Native American and colonial cultures. The leaves when roasted and boiled twice in water yield a black tea (Hume 1953). For centuries before colonial invasion, Native Americans used what they called yaupon tea as a social beverage, medicine, and emetic (Hudson 1979). It was the

third use, to induce vomiting in order to rid the body of contamination before council meetings, that inspired the species name of the plant. For much of the 17th century, prior to the advent of tea and coffee as important beverages in Europe, yaupon tea was an export to Spain, France and England (Hudson 1979). Its use was again popularized in the South during the Civil War when coffee and tea supplies were cut off.

Florida is in a sub-tropical zone of vegetation. The relative abundance of dioecious plant species compared to monoecious species in South Florida (27% of all plant species) approaches that of tropical forests of Costa Rica (21-29%) but is twice the number of temperate forests in the United States (6-17%) (Bawa and Opler 1975). Ilex vomitoria and possibly other hollies share two attributes of tropical dioecious plants relative to pollination.

While temperate dioecious plants are primarily wind pollinated (Stebbins 1951), tropical dioecious plants have been shown to be insect pollinated (Bawa and Opler 1975). The Aquifoliaceae are in the order Celestrales, which includes insect and wind pollinated families (Brizicky 1964). Hollies are found both in temperate and sub-tropical zones of the

United States and are probably insect pollinated throughout the family range. Cavigelli et al. (1986) argue for insect pollination of I. montana, a holly found from New York to Louisiana. Ilex cassine L. and I. krugiana Loess., both found in Florida, are insect pollinated (Tomlinson 1974). High numbers of insect visitations to I. vomitoria in Florida, especially Nematocera and Hymenoptera (personal observation), also implicate insect pollination for this holly species. The small size and abundance of I. vomitoria pollen, however, suggest the possibility of wind dispersal. Such dispersal may have no value for pollination, but may result in a pollen plume whose volatiles serve as attractants for insect visitors.

Temperate dioecious plants produce larger female flowers than male flowers (Baker 1948). The flowers of male and female I. vomitoria plants, however, are similar in size, a characteristic more typical of tropical dioecious plants (Bawa and Opler 1975).

Bawa and Opler (1975) observed a strong correlation between the timing of flowering in dioecious plants in Costa Rica forests and seasonal emergences of insect pollinators. In that study most insect visitors were small, with Hymen-

optera being the most likely pollinators. The flowering of I. vomitoria coincides with the spring population peak of C. mississippiensis. Few other flowering plants are as ubiquitous as I. vomitoria in March and April. Poison ivy, Toxicodendron radicans L., is the only other plant in flower at that time from which C. mississippiensis adults have been collected in vacuum samples.

Fine tuning of the relationship between pollinator and plant can be effected through the timing of release of nectar and pollen rewards (Janzen 1966). Prior to flowering, plants may encourage insect visitations by secretions of extrafloral nectaries. The Aquifoliaceae are not listed by Elias (1983) among plant families with extrafloral nectaries and none were seen on I. vomitoria by the investigator. Most flowers begin nectar production before pollinators arrive and stop once a maximum volume nectar pool is achieved (Cruden et al. 1983). Stiles (1975) suggests early morning as a good time for diurnally pollinated plants to present large amounts of nectar because production could occur throughout the night. However, cool nighttime temperatures may inhibit or delay nectar secretion in some plants (Cruden et al. 1983).

Red fruits develop on I. vomitoria and yield a maximum of four seeds. Diaz-Colon et al. (1970) determined that aqueous extracts of the fruit can inhibit root growth of other plant species' germinated seeds. Perhaps this capability gives I. vomitoria a competitive advantage over other plant species in colonization of new habitats. The fruit may be eaten by birds and seeds dispersed in their droppings. Germination and growth are slow, but more rapid population expansion can be achieved asexually by root-sprouting (Hume 1953).

The salt marsh habitat of Florida's Big Bend Gulf Coast region, from St. Petersburg to Pensacola, is an important breeding ground for C. mississippiensis. Preliminary vacuum samples of flowering I. vomitoria made in spring 1994 (Chapter 4) contained both adult male and female C. mississippiensis. Nectar sugars are a significant reward to C. mississippiensis adults for I. vomitoria floral visitations (Chapter 4). Attraction to flowers is mediated at least in part by floral volatile emissions (Metcalf and Metcalf 1992).

The purpose of this study was to characterize the I. vomitoria population of a salt marsh hammock in Yankeetown, Levy county, Florida to facilitate volatile sampling.

Specific objectives of the study were to map distribution of plants, determine spatial and temporal patterns of flowering, and select a representative group of plants for volatile collections.

Materials and Methods

Distribution Mapping

An aerial photograph taken by U. S. Air Force Tactical Reconnaissance Wing in 1981 (Figure 2-3) was used as a template for producing a scale map of the study site hammock, referred

to as hammock A (Figure 2-4). Four other neighboring hammocks were periodically visited for comparison to hammock A and were designated as hammocks B-E. Quadrats 10m on each side were assessed and plant positions plotted on the map of hammock A. Plants were considered as individuals if separated by at least one meter or if of different sex. Plants were tagged with pink surveyor's tape on which the hammock and code number were written. Each plant was sized as small (<1m high), medium (1-2m high) or large (>2m high).

Figure 2-3. Aerial view of salt marsh, Allen Park Road, Yankeetown, Florida. Study site hammock A and reference hammocks B-F indicated. Hammocks are in proximity to rich C. mississippiensis breeding grounds associated with Spartina alterniflora Loisel (Sa) and Juncus roemerianus Scheele (Jr).

Figure 2-4. Enlargement of hammock A, Allen Park Road, Yankeetown, Florida.

Spatial and Temporal Patterns of Flowering

Observations made in the spring of 1993 and 1994 indicated that the I. vomitoria flowering season begins in March and ends in April. In spring 1995 weekly observation of bud development was made starting in February. As soon as the first plant began flowering, complete surveys of hammock A I. vomitoria plants were made on an average of 2-3 day intervals. Date of first flowering and sex of each plant were noted. The data obtained were evaluated for sex ratio as well as spatial and temporal variations in bloom by sex across hammock A. Daily temperature readings were made with a Taylor alcohol thermometer during each of the four diel sample periods, corresponding to hours at sunrise, late morning, early afternoon, and sunset.

Sample Group Selection and Evaluation

The cost of materials and time required for movement between plants resulted in focusing the study on male I. vomitoria. Large male plants, up to a maximum of three plants at any given time, were included in volatile sampling. Candidate sample plants were identified before the start of flowering. Plants were brought into the study at the first sign of flowering and replaced when fully senesced. In some

cases it was necessary to replace a plant before flowering had completely stopped in order to accommodate inclusion of a plant just starting to flower.

Prior to the start of the flowering season the mean number of buds per centimeter of male stem was determined. Ten 5-cm-stem samples were randomly selected from each of three male plants with well-developed buds. All buds were counted using a dissecting microscope. Mean bud counts per centimeter of stem were determined for each plant and plant means examined by ANOVA and the REG-WQ (Ryan-Einot-Gabriel-Welsch Multiple Range Test) (SAS Institute, 1989).

Male bloom was quantified to make correlations between flowering phenology and volatile phenology. A male budded branch (sample segment) was enclosed in a tomato support cage. During each sample period, the number of open flowers of a sample segment was counted. Counting was facilitated by marking counted cymes with a small piece of masking tape. At the end of sampling, the total length of each plant's budded stems was measured. That length multiplied by the mean flowers per centimeter of stem gave the total flowers per sample segment. Percent bloom was calculated by dividing the number of open flowers in any given sample period by the total

number of flowers on the sample segment. Mean values for three day peak bloom were compared for the five plants by ANOVA and REG-WQ. Data for all plants included in the study were combined in a group bloom profile to statistically compare peak insect activity and percent bloom (Chapter 3).

Results

Distribution Mapping

Ilex vomitoria plant positions are shown on the map in Figure 2-5. A key to specific plant positions is given in Table 2-1. There are 145 *I. vomitoria* plants on hammock A, in an area of approximately 5600 m^2. Fifty-nine plants are female, 71 are male and 15 are of undetermined sex (did not flower). The sex ratio for Hammock A is 1.20:1. The data are presented in size groups in Table 2-2.

Spatial and Temporal Patterns of Flowering

The first plant to flower in Hammock A, starting on March 14, 1995, was a male coded A36. The last bloom start date, on April 15, was for a female plant coded A104. Bloom start dates covered a span of 33 days. Dates for the start of flowering of individual plants are given in Appendix A.

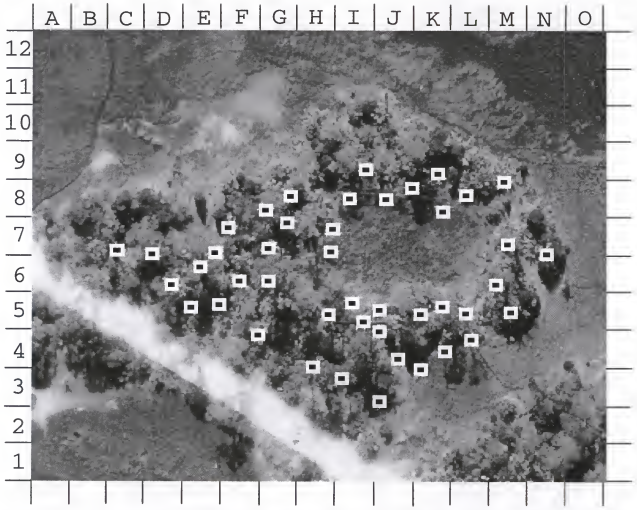


Figure 2-5. Hammock A showing positions of Ilex vomitoria in quadrats with sides of 10 m. Key to plants in each quadrat given in Table 3-1.

Table 2-1. Key to map of Figure 2-5, relating Ilex vomitoria plant designations to plotted positions on map.

Plant Designation	Map Location
1	C6/7
2,3	D6/7
4-11	E6
12	D6
13	E5
14	E6/7
15-21	F7
22,29-31	G6
23	F6
24-26	EF5
27,28	FG4/5
32,33	G6/7
34-36	G7/8
37	FG8
38,62-64	H7
39,40	H6/7
41,48,142	H5
42-49,135	I5
50-52,139	I3/4
53-55,143	IJ3
56-58,132	IJ4/5
59,61,145	J4
60,140	I5/6
65-69	H7
70-75	IJ8
76-78,144	I9
79,80	I8
81-83,85-87	J8/9
84	K9
88,89	K8
90	L8
91,92	M8/9
93-95	M7
96,97,141	N7
98-100	LM6
101-105	M5
106-110	L4

Table 2-1. continued

Plant Designation	Map Location
111-117	L5
118,119,124-126	K4
120-123	K3/4
127-130	K5
131	JK5
133,134	IJ5
135-138	G8

Table 2-2. Number of Ilex vomitoria plants on Hammock A in Yankeetown, Florida classed by size.

Sex	Size			Total
	Small	Medium	Large	
M	26	25	20	71
F	26	14	19	59
Unknown	14	0	1	15
Total	66	39	40	145

Phenology of start of flowering is shown in Figures 2-6.A-G. The mean start date for flowering of male plants was day 11, while that of female plants was day 18. The difference between male and female start dates was significant (ANOVA, $F=109.0$, $df=11,118$, $p<0.0001$).

When size groups are considered individually, large plants are shown to flower earlier than medium plants, which flower earlier than small plants. A statistically significant difference for start of flowering exists between large and small male plants, and between all male plants, except small, and female plants (ANOVA, $F=13.51$, $df=5,124$, $p<0.0001$). Mean start dates for large, medium and small male plants were 9, 11, and 13 days, respectively. For large, medium and small female plants mean start dates were 16, 17 and 19 days, respectively. No statistical differences were observed between female size groups relative to the date for start of flowering. A significant interaction existed for start of flowering with the mean daily temperature calculated from daily high and low temperature readings. A graph for the start of flowering by sex is shown in Fig. 2-7.

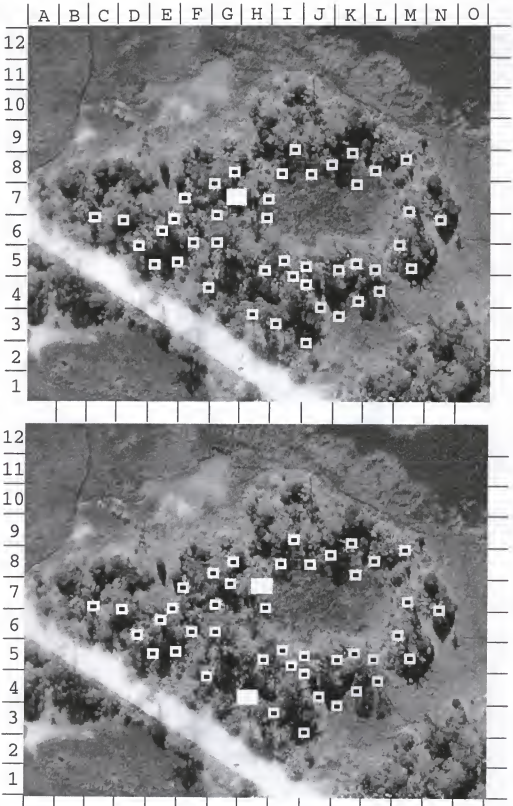


Figure 2-6A. Phenology of flower start for I. vomitoria on hammock A. Start of bloom indicated by white square. Top is for 3/14/95, bottom for 3/16/95.

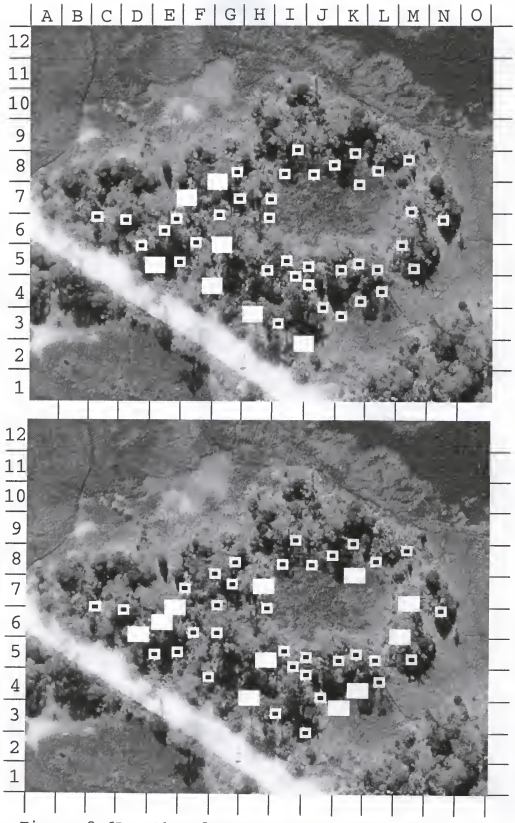


Figure 2-6B. Phenology of flower start for I. vomitoria on hammock A. Start of bloom indicated by white square. Top is for 3/18/95, bottom for 3/21/95.

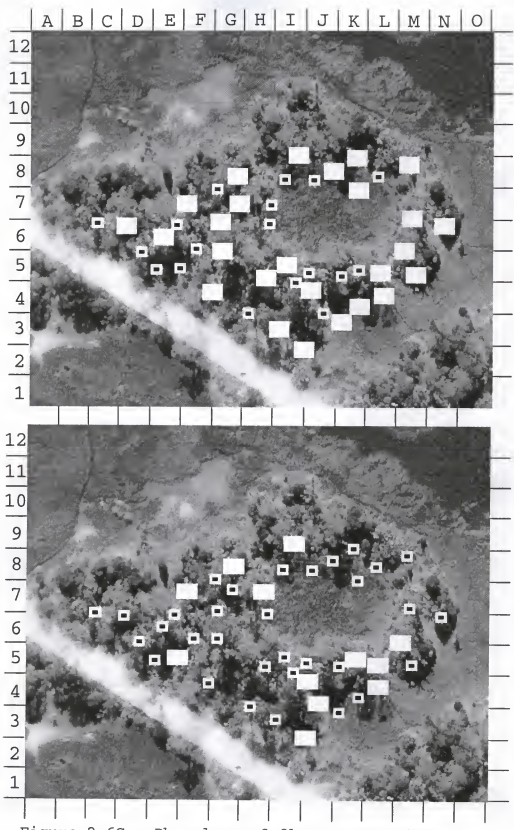


Figure 2-6C. Phenology of flower start for I. vomitoria on hammock A. Start of bloom indicated by white square. Top is for 3/24/95, bottom for 3/27/95.

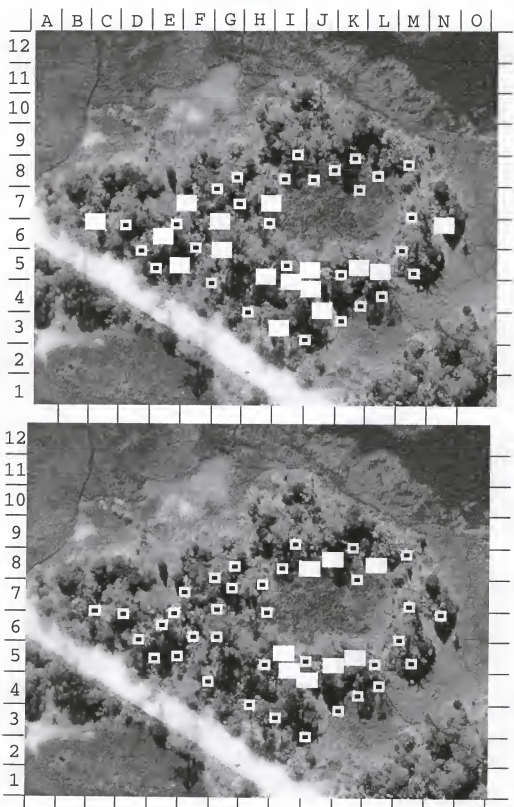


Figure 2-6D. Phenology of flower start for I. vomitoria on hammock A. Start of bloom indicated by white square. Top is for 3/29/95, bottom for 3/31/95.

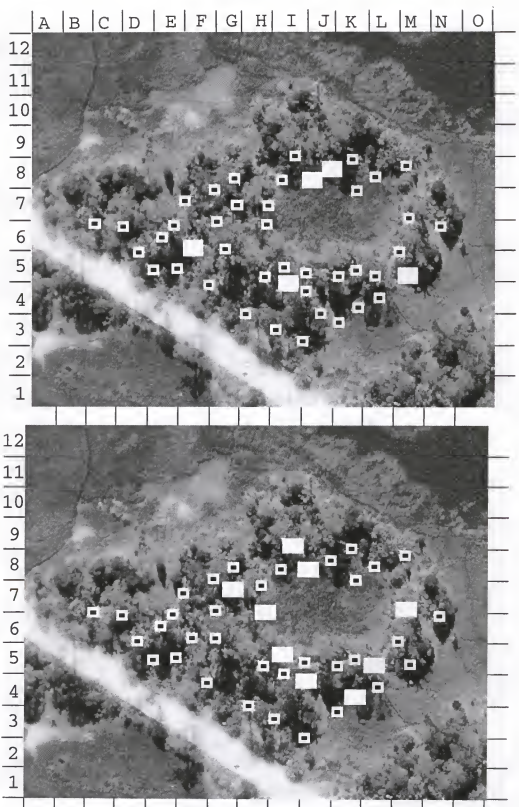


Figure 2-6E. Phenology of flower start for I. vomitoria on hammock A. Start of bloom indicated by white square. Top is for 4/3/95, bottom for 4/7/95.

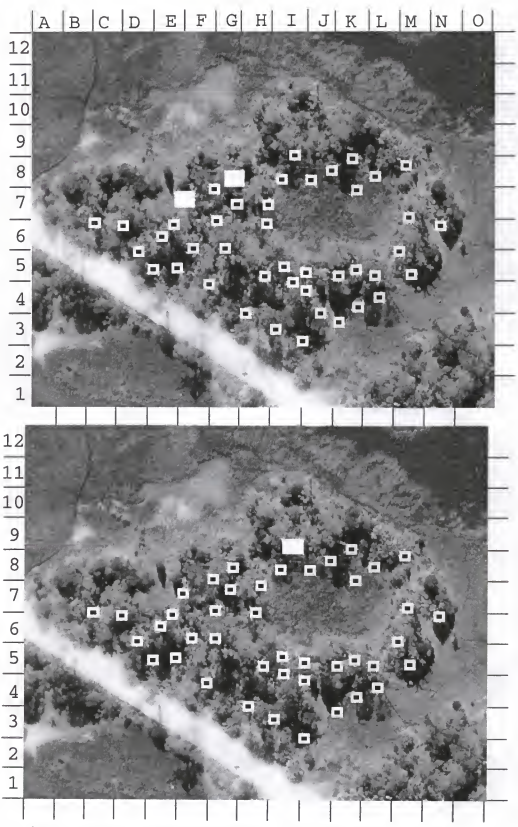


Figure 2-6F. Phenology of flower start for I. vomitoria on hammock A. Start of bloom indicated by white square. Top is for 4/9/95, bottom for 4/12/95.

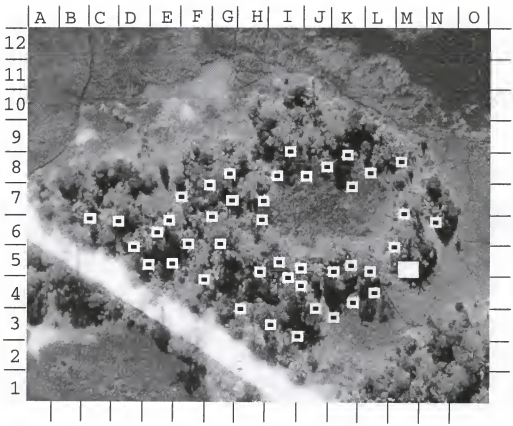


Figure 2-6G. Phenology of flower start for I. vomitoria on hammock A. Start of bloom indicated by white square. Above is for 4/15/95.

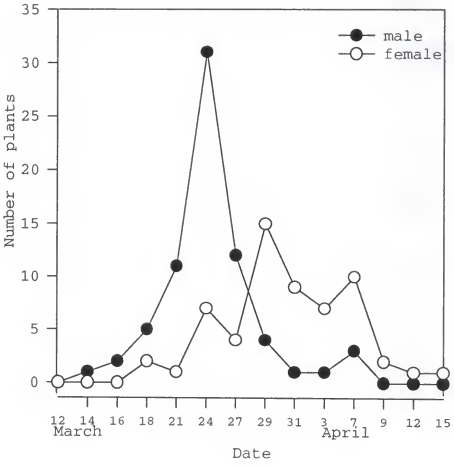


Figure 2-7. First day of flowering by sex. Ilex vomitoria on Hammock A, Allen Park Road, Yankeetown, Levy Co., March-April 1996.

Sample Group Selection and Evaluation

Plants included in the study are listed with dates of flowering in Table 2-3. A total of five large male plants were sampled.

Results of bud count data are given in Table 2-4. All means were evaluated by ANOVA and REG-WQ multiple comparison test. Male means are not significantly different from each other (ANOVA, $F=0.17$, $df=2,27$, $p=0.85$), and so the overall mean for males is valid. Female means do differ significantly (ANOVA, $F=25.8$, $df=2,27$, $p<0.0001$), with each mean shown by multiple comparison to be significantly different. Male mean bud count per centimeter stem is significantly greater than female mean bud count (ANOVA, $F=100,000$, $df=6,53$, $p<0.0001$). The mean value of 20.4 buds/cm was used to evaluate percent bloom of male plants included in the study.

Data for calculation of percent bloom is found in Appendix B. Percent bloom for each of the five study plants is shown in Figure 2-8 A to E. Combined percent bloom data for the study group was stratified into 8 groups for later statistical examination of the relationship between insect visitations and flowering status (Chapter 3). The 8 groups were constructed to include more than 15 data values each

Table 2-3. Ilex vomitoria plants included in the study by hammock code designation.

Plant	Sex	Flowering dates
A36	M	3/16-3/31
A14	M	3/24-4/7
A62	M	3/25-4/7
A35	M	4/1-4/15
A129	M	4/7-4/15

Table 2-4. Mean bud counts cm^{-1} (SE) for I. vomitoria in March 1995. Ten 5-cm-segments of budded stem from each of 3 male and 3 female plants were evaluated.

	Sex	
Plant	Male	Female
1	21.6 (2.61)	2.5 (0.40)
2	20.1 (3.56)	1.5 (0.28)
3	19.3 (1.85)	4.8 (0.31)
Overall mean	20.4 (1.55)	2.9 (0.33)

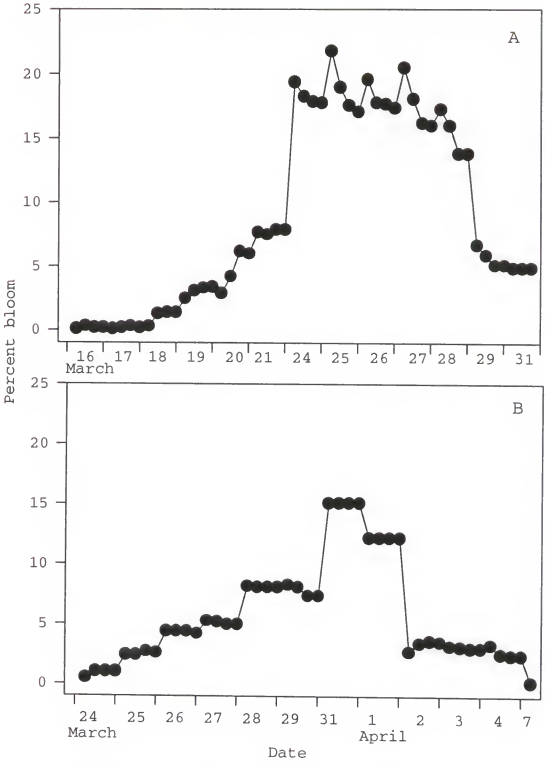


Figure 2-8. Graph of percent bloom as a function of time, throughout the flowering season.
A. Profile for plant A36.
B. Profile for plant A14.

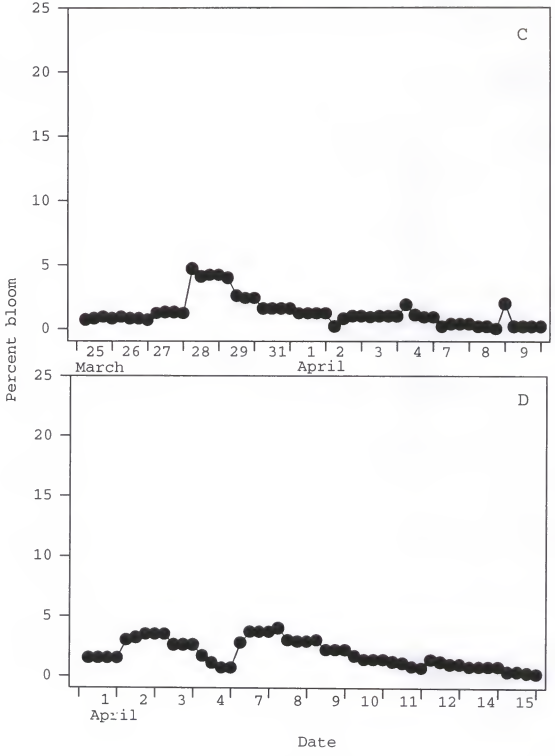


Figure 2-8. Graph of percent bloom as a function of time, throughout the flowering season.
C. Profile for plant A62.
D. Profile for plant A35.

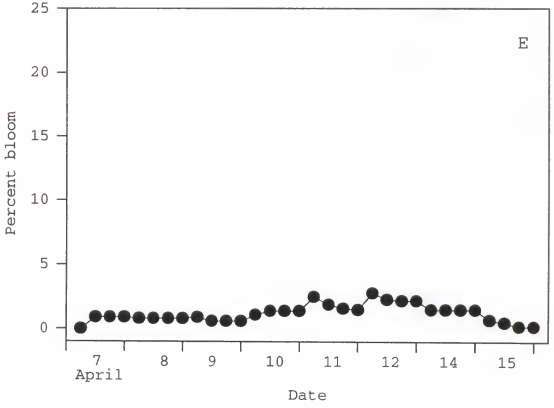


Figure 2-8. Graph of percent bloom as a function of time, throughout the flowering season.
E. Profile for plant A129.

and to reflect the flowering cycle (increase to peak followed by decline). Those groups are shown in Table 2-5.

Statistical comparison of the five plants of the study group was made relative to the three days of highest percent bloom for each (peak bloom). Peak bloom declined significantly over the flowering season. Plant A36, the first plant to flower, had a significantly higher peak bloom than plant A14, and both A36 and A14 were significantly higher than the other three plants in the study (ANOVA, $F=271.1$, $df=14,45$, $p<0.0001$). Peak bloom means are summarized in Table 2-6.

Discussion

Distribution Mapping

The positions of *I. vomitoria* as shown on the map in Figure 2-5 are evenly scattered across hammock A. Although male plants outnumber female plants, no irregular distribution patterns relative to sex were detected. It should be noted, however, that formal analysis of spatial association was not carried out.

Sex ratio for hollies may be skewed in favor of males. Cavigelli et al. (1986) found a male:female ratio of 1.35:1

Table 2-5. Percent bloom (B) groups. Eight groups were constructed to reflect natural flowering cycle dynamics.

Group	B(% bloom)	n*
1	B<1	30
2	1≤B<2	17
3	2≤B<5	27
4	5≤B<22	28
5	22>B≥5	18
6	5>B≥2	32
7	2>B≥1	27
8	1>B	33

* Number of samples included in each bloom group.

Table 2-6. Mean peak bloom. Means are calculated for highest percent bloom over three days for each plant included in the volatile study. Values of all four diel periods are used for each day.

Plant	N	Mean	SE
A36	12	18.5A*	0.38
A14	12	10.1B	0.94
A35	12	3.02C	0.18
A62	12	2.88C	0.18
A129	12	1.92D	0.13

*Means followed by different letters are significantly different at the 0.05 probability level by REG-WQ multiple comparison test.

for I. montana Torr. & Gray. Richards' (1988) study of four natural or seminatural plots of I. aquifolium L. in Britain showed male:female sex ratios ranging from 1.11:1 to 2.16:1. The male:female sex ratio of I. opaca Aiton was determined to be 1.03:1, seeming to contradict the male-skewed trend of other hollies (Clark and Orton, 1967). However, the study of Clark and Orton was done on seedlings which were followed over a seven-year period in controlled conditions and does not reflect differential survival that may exist in a natural stand of I. opaca.

Two characteristics of hollies tend to complicate sex ratio determination. Their ability to reproduce vegetatively from roots makes it difficult to distinguish close growing same sex plants as truly unique individuals. The sex ratio of I. vomitoria is lower than that found for I. montana (Cavigelli et al. 1986), but is consistent with a seemingly male-skewed sex ratio for holly species. Several hypotheses to explain male-biased sex ratios in hollies are discussed by Cavigelli et al. (1986). The hypothesis favored is that female energetic costs are higher than male costs, resulting in reduced frequency of flowering. Therefore long-term studies should reveal a greater number of any year's non-

flowering plants to be female and the overall sex ratio to be close to 1:1. Studies carried out over several years in Hammock A could determine if this is the case for I. vomitoria.

Spatial and Temporal Patterns of Flowering

In their study of tropical dioecious plants, Bawa and Opler (1975) determined that male flowers open earlier in the day than female flowers. The self-incompatibility of such plants would favor out crossing, resulting in a population with greater genetic variation. Ilex vomitoria also shows temporal segregation of male and female floral rewards, with male plants producing flowers earlier in the season than female plants. This arrangement results in pollinator visitation of male plants before female plants. The advantage for the plant of such a system is to increase the chance a pollinator will visit a female flower bearing pollen from an unrelated plant.

Sample Group Selection and Evaluation

The choice of conducting the study on male rather than female plants was based in part on the potential for greater levels of flowering. Plants with more flowers would provide greater quantities of floral volatiles as well as insects.

Bud counts that showed significantly greater numbers in male than female plants supported the choice of male plants. Male plants were also shown to begin flowering significantly earlier than female plants. Choice of male plants therefore allowed earliest start of insect sampling, yielding a more complete profile of insect visitation throughout the flowering season.

The level of percent bloom achieved by plants through the flowering season varied from high early in the season to low late in the season. It is difficult to judge how much the low bloom level achieved by A62, A35 and A129 was the product of thrips infestations and how much was a normal end of season phenomenon. Damage to buds caused by high thrips populations may be at least in part responsible for considerably lower percent blooms of plants. This explanation is supported by data presented in Chapter 3 showing that highest numbers of thrips in collections were significantly associated with lowest percent bloom.

CHAPTER 3
ILEX VOMITORIA FEEDING GUILD

Introduction

The Diptera genus Culicoides Latreille, 1909 includes a large number of species of extremely small biting flies with worldwide distribution. They are classified in the suborder Nematocera, family Ceratopogonidae, subfamily Ceratopogoninae and tribe Culicoidini (Wirth et al. 1980). Culicoides spp. are important pests and disease vectors. Males feed on the nectar of flowers, while females feed on blood. The genus shows great diversity of blood source preference, but most feed on mammals and birds (Downes 1970). In many species autogeny, that is the production of the first batch of eggs following a sugar meal, provides an adaptive advantage in habitats where blood meals may not always be available (Downes 1970).

Worldwide there are more than 1,000 species of Culicoides (Wirth et al. 1980). In North America there are 144 described

Culicoides species, of which 48 can be found in Florida (Blanton and Wirth 1979). As many as 21 of those species have been found in Yankeetown, Florida (Kline 1986).

Adult Culicoides can be distinguished from other ceratopogonids by having a 3-segmented antenna, including the flattened scapes (segment 1), enlarged pedicel (segment 2) and flagellum (segment 3), which is subdivided into 13 flagellomeres. Wings are characteristically patterned with light spots on a dark background. These patterns, as well as antennal ratios, the number and type of antennal sensilla, and male terminalia are important taxonomic characters for species identification (Wirth, personal communication). Species identifying criteria for Florida species are outlined by Blanton and Wirth (1979).

The four most common anthropophilic species in Yankeetown are C. mississippiensis Hoffman, C. furens Poey, C. barbosai Wirth & Blanton, and C. floridensis Beck (Lillie et al. 1988). While C. mississippiensis is bivoltine, with spring and fall population peaks, the other three species are most often found from April to October when C. mississippiensis begin to decline and reach their lowest levels. Host seeking is characterized as crepuscular in C. mississippiensis, C.

barbosai and C. floridensis but nocturnal in C. furens (Lillie et al. 1988).

Culicoides mississippiensis has been shown to be autogenous (Davis 1981). No sugar sources have been identified prior to the present study although midge vacuum samples by Lillie and Kline (1986) suggested flowering Ilex vomitoria Aiton, the yaupon holly, as a possible sugar source in spring. An important first step in studying the association between C. mississippiensis and I. vomitoria is to establish the nature of the reward of attraction to the flowering plants. It may seem reasonable to assume that the insect visits are linked to sugar feeding, but determination of such a link should be made by conducting anthrone tests.

The success of many nematoceran pests and disease vectors is linked to autogeny and sugar feeding. Sugar feeding not only fuels initial egg production but also provides energy for flight (Van Handel 1984), enabling search for blood meal hosts and oviposition sites, and increases longevity (Hunter 1977, Jamnback 1961). Lack of contact with humans and alternate hosts may favor higher sugar feeding rates (Edman et al. 1992, Van Handel et al. 1994). Implications for control of protozoan vectors in sylvan cycles are significant given

that sugar meals increase transmission rates, possibly by nourishing developing parasites (Young et al. 1980).

The cold anthrone test (Van Handel 1972) is the primary tool used in studying sugar feeding behavior of insects. Crushing a sugar fed insect in yellow anthrone solution will cause the reagent to change to color shades ranging from light green to dark blue. Mosquitoes (Bidlingmayer and Hem 1973, Edman et al. 1992, Reisen et al. 1986, Smith and Kurtz 1994, Van Handel et al. 1994), black flies (McCreadie et al. 1994, Walsh and Garms 1980, Young et al. 1980), and biting midges (Magnarelli 1981, Mullens 1985) are the principal nematocerans on which anthrone testing has been used.

Nectar is considered a primary attractant of insects to flowers (Faegri and Pijl 1979). It contains sugars, amino acids, lipids, anti-oxidants, and vitamins (Inouye 1980). In addition to being important components of the nectar reward, sugars and amino acids may also act as attractants themselves (Baker and Baker, 1986). Nectar glycerides that fluoresce may also attract insects (Kevan 1976).

Flowers have been shown to emit a large number of volatile compounds which serve to attract insects. In some cases the volatiles act as mimics of insect glandular

secretions (Borg-Karlson and Groth 1986). Some pollens produce their own set of volatiles that are important insect attractants (Pijl 1960, Dobson et al. 1987). Perfumes, UV-reflecting nectar guides, and visible color guides act as close range attractants to insects (Snyder and Miller 1972). Establishment of the nectar of I. vomitoria as a sugar source for male and female C. mississippiensis would provide a rationale for examining floral volatiles as possible attractants.

Anthrone tests made on other Culicoides species (Magnarelli 1981, Mullens 1985) confirmed prior sugar feeding by host-seeking females, but did not identify sugar sources. Such data may be of limited value for determining circadian feeding rhythms and population feeding rates because anthrone negatives and weak positives could include insects that fed some time before sampling (Walsh and Garms 1980, Reisen et al. 1986, Smith and Kurtz 1994). Insect samples taken directly from flowering plants would provide more reliable information since the time of collection is more directly related to the opportunity for sugar acquisition.

An ecologically important concern that must be addressed before control of a pest is attempted is the impact such control may have on non-target arthropods. If control of C.

mississippiensis is to be based on its association with flowering male I. vomitoria, then other insects that visit the flowering plants must be identified and temporal patterns of floral visitations evaluated. Identification of floral attractants that correlate to peak foraging by target insects, but not to non-target insects, may facilitate selection of chemicals to be tested for response by insects.

The objectives of the following study were to identify the principal insects present on flowering male I. vomitoria, both before and during the flowering season, characterize the periodicity of their activity relative to the time of day and time within the flowering season and establish the nectar feeding behavior of C. mississippiensis. A comparison of the nectar and blood feeding of C. mississippiensis was also made.

Materials and Methods

Non-flowering Samples

Samples of insects were collected from three male I. vomitoria prior to the start of the flowering season at the Yankeetown study site using an AFS sweeper (Meyer et al. 1983). The sweeper is a backpack vacuum device with a

flexible extension arm that holds a collection container. Insects were collected in cardboard pint containers fitted with 100 mesh screen hot-glued into top and bottom covers.

Plants sampled were A14, A36 and A39. Samples were made by vacuuming vegetation for one minute. Vacuum sweeps were made away from the collector to avoid influencing sample contents. Samples were collected on 5 March, 11 days before the start of the flowering season, at noon and sunset, and on 14 March, 2 days before flowering, at sunset only.

At completion of sampling, containers were closed and placed inside zipper-top plastic bags for insect anaesthetization by CO_2. Insects were then transferred to vials and held on wet ice until return to Gainesville. Cooling of samples before processing was needed to prevent spiders from feeding on insects. All collected arthropods were freeze-killed and preserved in 70% ethanol for later processing.

Totals were recorded for major taxa. Mean number per minute sample and standard error were calculated.

Flowering Season Samples

Five flowering male I. vomitoria were vacuum-sampled with an AFS sweeper four times a day throughout the flowering season of the holly. Plants sampled (dates of sampling)

included A36 (16-31 March), A14 (24 March-7 April), A62 (25 March-9 April), A35 (1-15 April) and A129 (7-15 April). One-minute AS sweeper samples were collected and handled according to the protocol for non-flowering season samples. The diurnal portion of the diel cycle was divided into ten periods and samples were made during the first, fourth, seventh and tenth periods, corresponding to sunrise, late morning, early afternoon and sunset. Sampling was limited to the diurnal portion of the diel cycle because C. mississippiensis does not feed at night (Lillie et al. 1988). Nighttime sampling may have yielded a more complete picture of the I. vomitoria feeding guild as well as the phenology of essential oil emission, but was not carried out.

Most male and female C. mississippiensis were removed from samples and stored separately at -60°C until processed by the cold anthrone test. All other sample contents, including some C. mississippiensis, were preserved in 70% ethanol and processed at a later date.

Preserved sample contents were sorted according to general taxa and subsequently as many identified to species as possible. The total number of all invertebrates collected was recorded for each plant by diel period for each sample day of

the flowering season. Mean number per minute sample and standard error were calculated and general evaluations made for all taxa except C. mississippiensis and the two dominant insects, Dasyhelea mutabilis Kieffer and thrips. Those three taxa were submitted to more detailed analysis of temporal visitation rhythms.

Visitation Rhythms

Sample data were analyzed to identify any daily or seasonal rhythmic patterns of visitation by C. mississippiensis to flowering yaupon. Detailed analysis of visitations by D. mutabilis and thrips was also made because they would probably be the most abundant non-target victims. Graphs were made for each of those insects of the numbers collected by plant and for the entire flowering season. Mean (standard error) one-minute samples by diel period were calculated to identify diurnal visitation patterns. Those means were examined by ANOVA and significant differences identified by REG-WQ.

Visitation relative to flowering season was also analyzed. Insect means for each plant were compared by ANOVA and REG-WQ to identify visitation patterns relating to the entire flowering season. Plants were sampled sequentially in

an order that can be considered to represent early (A36), mid (A14, A62), and late (A35, A129) flowering season.

Bloom groups were described for I. vomitoria male plants in Chapter 2 (Table 2-5). These bloom groups (BG) correspond to stages in flowering that may be referred to as initiation (BG1,2), expansion (BG3,4), decline (BG5,6) and senescence (BG7,8). An analysis of visitations by bloom group was made comparing the mean number of insects captured for each of the eight bloom periods described. Means were examined by ANOVA and significant differences identified by REG-WQ multiple comparison.

Anthrone tests

Anthrone tests were run on a stratified random sub-sample of males and gravid females. Gravid females could be easily distinguished from the few non-gravid females in samples by their swollen, lighter pigmented abdomens. Midges were assigned chronological numbers according to the date and time of sampling. Random number tables were used to select midges for anthrone testing, with numbers proportionately allotted to the four sample time periods for each sex. The estimate of nectar feeding rate was made using a 90% confidence interval and sample size calculated by the confidence interval formula.

Because $\hat{p}$ was unknown, a value of 0.5 was used to calculate the sample size. By this method, 384 males and 384 females were tested to obtain a 90% confidence interval. Parous females taken in samples were also tested.

Fresh anthrone reagent was made following the method of Van Handel (1972) every two to three days . Selected insects from each cohort were rinsed in distilled water to remove any nectar, which may have adhered to them during vacuuming, and placed individually in 10 x 75 mm test tubes. Midges were then crushed with a glass stirring rod in 0.2 ml anthrone reagent and evaluated one hour later for color change. Results were reported as positive or negative. The stirring rod was rinsed in two beakers of water and then dried with a paper towel. Controls were made by mixing the stirring rod in anthrone reagent alone after every six insect tests. Lack of available known negative insects, due to low field emergence levels at the time of testing, precluded better experimental control. Data was analyzed by chi-squared tests.

Biting rates of C. mississippiensis

Midge biting rates were determined for the same four sample periods from 16 March to 15 April. Midges were aspirated from the left forearm and numbers recorded every 60

seconds for 20 consecutive 60 second periods. When densities exceeded 20 bites/60sec, a 5 min aspiration sample was made. During the next fifteen minutes midges were killed, but not collected, as they were counted. Samples were later identified to species.

Results

Non-flowering Samples

A total of nine AS sweeper samples were collected from non-flowering male I. vomitoria. The composition of the samples is given by summary taxa in Table 3-1.

Very low numbers of insects for major taxa were present on plants that had not yet flowered. In particular, the mean numbers of thrips, D. mutabilis, and C. mississippiensis were less than 2 each per sample. No taxon dominated samples and most taxa had less than 10% of the total sample contents.

Flowering Season Samples

A total of 218 AS sweeper samples were collected from flowering I. vomitoria between 16 March and 15 April 1995. Sample contents are given by summary taxa in Table 3-2.

Table 3-1. Invertebrates collected from Ilex vomitoria prior to the start of flowering. Samples (n= 9) from plants A36, A14 and A39 collected by AFS sweeper on 5 March and 14 March 1996.

Taxa	Non-flowering totals (%)	Non-flowering means (SE*)
Thysanoptera	15 (9.7)	1.7 (1.1)
Dasyhelea mutabilis (male)	15 (9.7)	1.7 (1.2)
D. mutabilis (female)	14 (9.0)	1.6 (1.1)
Culicoides mississippiensis (male)	0 (0.0)	0.0 (0.0)
C. mississippiensis (female)	12 (7.7)	1.3 (0.5)
Other Nematocera	19 (12.3)	2.1 (0.7)
Hymenoptera	18 (11.6)	2.0 (0.7)
Collembola	15 (9.7)	1.7 (0.5)
Hemiptera	3 (1.9)	0.3 (0.3)
Coleoptera	2 (1.3)	0.2 (0.2)
Other Diptera	9 (5.8)	1.0 (0.7)
Acari	3 (1.9)	0.3 (0.2)
Lepidoptera	6 (3.9)	0.7 (0.2)
Homoptera	10 (6.5)	1.1 (0.8)
Araneida	9 (5.8)	1.0 (0.3)
Psocoptera	2 (1.3)	0.2 (0.2)
Mollusca	3 (1.9)	0.3 (0.2)

* Standard error

Table 3-2. Invertebrates collected from Ilex vomitoria during the entire flowering season. Samples (n= 218) from plants A36, A14, A62, A35 and A129 collected by AFS sweeper from 16 March and 15 April 1996.

Taxa	From Flower totals (%)	From Flower means (SE*)
Thysanoptera	65504 (59.5)	299.1 (30.7)
Dasyhelea mutabilis (male)	16223 (14.7)	74.1 (8.0)
D. mutabilis (female)	17469 (15.9)	79.8 (7.8)
Culicoides mississippiensis (male)	2459 (2.2)	11.2 (1.2)
C. mississippiensis (female)	1332 (1.2)	6.1 (0.5)
Other Nematocera	2394 (2.2)	10.9 (0.9)
Hymenoptera	1902 (1.7)	8.7 (0.6)
Collembola	796 (0.7)	3.6 (0.4)
Hemiptera	697 (0.6)	3.2 (0.2)
Coleoptera	323 (0.3)	1.5 (0.1)
Other Diptera	287 (0.3)	1.3 (0.1)
Acari	222 (0.2)	1.0 (0.1)
Lepidoptera	143 (0.1)	0.7 (0.07)
Homoptera	137 (0.1)	0.6 (0.07)
Araneida	122 (0.1)	0.6 (0.07)
Psocoptera	23 (<0.1)	0.1 (0.02)
Mollusca	22 (<0.1)	0.1 (0.02)

* Standard error

Specific identifications and relative occurrence are shown in Table 3-3.

More than 90% of all arthropods collected were either thrips or D. mutabilis. Culicoides mississippiensis comprised 3.4% of the sample total and was the third most abundant arthropod on flowering yaupon. Non-arthropods were very uncommon and included anoles, observed feeding on insects, and small birds. Arthropod visitors may be generally grouped into three categories: uncommon (less than 1% of the sample total), common (between 1 and 10% of the sample total) and very common (more than 10% of the sample total). General analysis for uncommon and common visitors, except C. mississippiensis, follow. More detailed analysis of data for C. mississippiensis and the two very common insect taxa is given separately in the section on temporal visitation patterns.

Uncommon visitors

Psocoptera (<0.1%). No sample had more than two psocids. Samples containing psocids were few and scattered throughout the flowering season.

Araneida (0.1%). Spiders could be found at low levels throughout the flowering season, with an unusually high number (12) taken from A35 during diel period 1 on April 2.

Table 3-3. Arthropods identified from AS sweep samples of Ilex vomitoria, spring 1995. Occurrence is indicated by +++ (very common), ++ (common), and + (uncommon).

Taxa	Occurrence
Arachnida	
Acaridae	
Trombiculidae	+
Araneae	
Anyphaenidae	
Habana sp.	+
Araneidae	
Argiope sp.	+
Metazygia sp.	+
Neoscona sp.	+
Salticidae	
Hentzia sp.	+
Theridiidae	
Anelosinus sp.	+
Coleosoma sp.	+
Insecta	
Collembola	
Entomobriidae	+
Coleoptera	
Curculionidae	+
Oedemeridae	+
Diptera	
Ceratopogonidae	
Culicoides barbosai Wirth & Blanton	+
C. furens (Poey)	+
C. mississippiensis Hoffman	++
Dasyhelea mutabilis Kieffer	+++
Dasyhelea sp.	+
Forcipomyia sp.	+
Cecidomyidae	+
Chironomidae	+
Mycetophilidae	+
Sciaridae	+

Table 3-3 (continued)

Taxa	Occurrence
Hemiptera	
Lygaeidae	+
Homoptera	
Aphididae	
Toxoptera aurantii (Boyer de Fonscolombe)	+
Cixiidae	
Pintalia vibex Kramer	+
Myndus sp.	+
Psyllidae	
Gyropsylla ilicis (Ashmead)	+
Aleyrodidae	+
Hymenoptera	
Eulophidae (4 spp.)	++
Mymaridae (1 sp.)	++
Torymidae (2 spp.)	++
Lepidoptera	
Geometridae	+
Lycaenidae	
Callophrys gryneus (Hübner)	+
Nymphalidae	
Agraulis vanillae (L.)	+
Thysanoptera	
Thripidae	
Frankliniella bispinosa (Morgan)	+++
Leptothrips sp.	+
Heterothrips sp.	+

Homoptera (0.1%). These insects were few and scattered throughout the flowering season. The most often encountered Homoptera included Toxoptera aurantii (Boyer de Fonscolombe) and Gyropsylla ilicis (Ashmead), the yaupon psyllid. All whiteflies in samples were adults.

Lepidoptera (0.1%). All lepidoptera collected from flowering yaupon were Geometrid larvae. Damage to leaves caused by grazing larvae, though not extensive, could be seen on many of the yaupon plants in the study site. Cumulative totals were greater than 50% at the end of two weeks and greater than 90% at the end of three weeks. More than 85% of all larvae were collected between 24 March and 8 April. The only adult Lepidoptera observed infrequently feeding on male yaupon flowers were the cedar hairstreak, Callophrys gryneus (Hübner), and the Gulf fritillary, Agraulis vanillae (L.).

Acari (0.2%). Mites found on flowering yaupon were of two major groups, detrivores and parasites. Several trombiculiids were found clinging to the abdomens of D. mutabilis collected on flowering yaupon.

Diptera (Brachycera and Cyclorrhapha) (0.3%). Larger Diptera were rarely trapped in AS sweeps but were occasionally observed at low numbers to be feeding from flowers. Most

frequently observed were syrphids (Syrphidae) and deer flies (Tabanidae). Most non-ceratopogonid Diptera visiting flowers were small cyclorrhaphan flies. Highest numbers occurred in two distinct phases, two days in late March (26-27 March) and nine days in April (2-12 April). Specimens collected on those eleven days accounted for more than 80% of all non-ceratopogonid Diptera.

Coleoptera (0.3%). Coleoptera were collected in relatively low numbers throughout the flowering season. Some, especially the oedemerids, were observed feeding on pollen.

Hemiptera (0.6%). The majority of bugs collected from flowering yaupon were lygaeids (Lygaeidae). Lygaeid numbers increased steadily from 25 March to 7 April. Both adults and immatures were found at all times of the day on plants.

Collembola (0.7%). Nearly all Collembola collected were of the family Entomobryidae. Their numbers increased to a peak of 103 on 27 March and then remained at stable but lower levels to the end of the flowering season. Higher numbers were found in diel period 1 samples than all other samples.

Common visitors

Hymenoptera (1.7%). Bees and wasps are important visitors of flowering yaupon holly. Although relatively few in

number, megachilids and vespids could be seen carrying copious amounts of pollen as they visited both male and female plants. Vespids were also frequently seen searching in yaupon foliage.

The majority of Hymenoptera in AS sweep samples were very small members of Chalcidoidea families. Species most commonly collected from flowering yaupon were of the Eulophidae, Torymidae, and Mymaridae. Over the entire flowering season significantly fewer Hymenoptera visits occurred during diel period 1 than the other three collection periods. Collections made during diel periods 7 and 10 had significantly higher numbers of Hymenoptera than earlier collections. Nearly two-thirds of all Hymenoptera collected were in samples of the last 11 days of the flowering season.

Nematocera (excluding C. mississippiensis and D. mutabilis) (2.2%). Nematocera were the second most abundant group of insects on flowering yaupon. Separate consideration is given to C. mississippiensis because of their relatively high numbers in samples. Other ceratopogonids were also found in important numbers, including Dasyhelea species and Forcipomyia species. Only two other species of Culicoides were collected, those being C. barbosai Wirth & Blanton and C. furens (Poey), both in very low numbers. Additional families

significantly represented in samples included Cecidomyidae, Chironomidae, Mycetophilidae, and Sciaridae.

Culicoides mississippiensis (3.4%). These ceratopogonid insects were the third most abundant insects in AS sweep samples of flowering I. vomitoria in 1995. Culicoides mississippiensis made up 95.7% of all biting midges collected, while 4.1% were C. barbosai and 0.2% C. furens. A total of 3,791 C. mississippiensis adults were collected, of which 2,459 (2.2%) were male and 1,332 (1.2%) female. Higher numbers of males than females is in agreement with expected feeding differences. Detailed analysis of C. mississippiensis data follows under visitation rhythms.

Very common visitors

Dasyhelea mutabilis (30.6%). This common ceratopogonid gnat was the second most abundant insect in 1995 samples. A total of 33,692 D. mutabilis adults, of which 16,223 (14.7%) were male and 17,469 (15.9%) female, were collected. Detailed analysis of D. mutabilis data follows under visitation rhythms.

Thysanoptera (59.5%). Thrips were the most abundant insects in samples of flowering I. vomitoria. Holes, made by thrips, could be seen in numerous buds of plants A62, A35 and

A129. Total bloom of those three plants was far less than of A36 and A14, which were not infested. The vast majority of thrips collected were Frankliniella bispinosa (Morgan). Both adults and immatures were collected from flowering yaupon. Detailed analysis of thrips data follows under visitation rhythms.

Visitation Rhythms

Culicoides mississippiensis. The combined total of males and females collected for each plant by diel period are shown in graphs of Figure 3-1. Fluctuating numbers reflect both daily and seasonal rhythms.

Daily rhythms. Combined data from all plants were analyzed by GLM and REG-WQ multiple comparison to identify diel foraging preferences. Mean (standard error) values for each diel period are shown in Table 3-4. Diel 10 had a significantly greater number of biting midges in collections than either diel 7 or diel 4, but was not significantly greater than diel 1.

Seasonal rhythms. Combined data was analyzed by GLM and REG-WQ multiple comparison to identify seasonal patterns of C. mississippiensis visitations to flowering I. vomitoria. Results of this analysis are given in Table 3-5. Mean

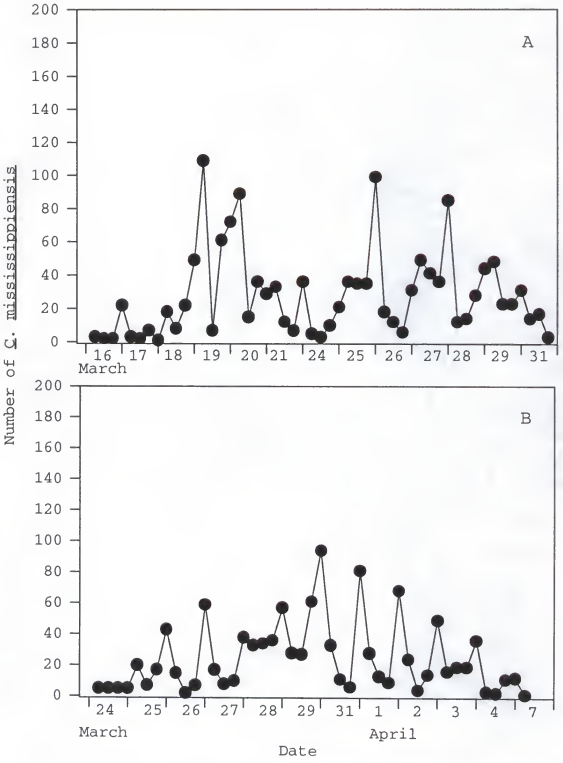


Figure 3-1. Number of Culicoides mississippiensis collected from Ilex vomitoria by diel period (sunrise, late morning, early afternoon, sunset).
A. Samples from plant A36.
B. Samples from plant A14.

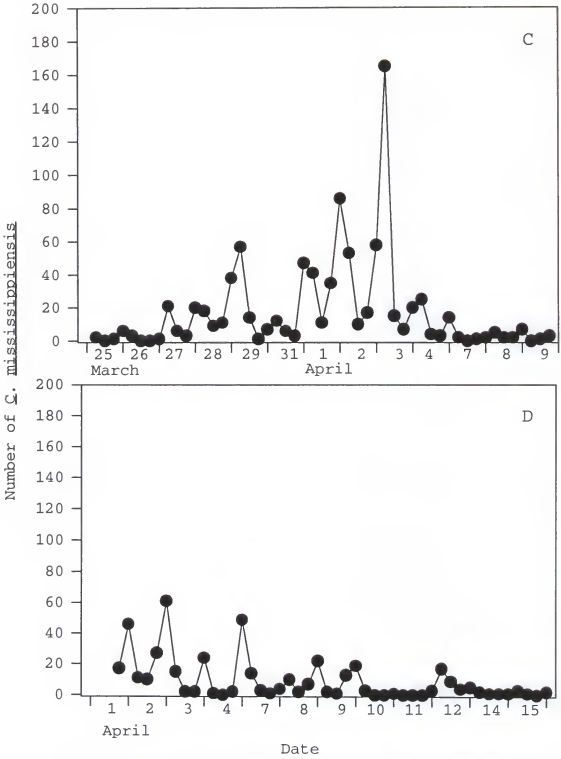


Figure 3-1. Number of Culicoides mississippiensis collected from Ilex vomitoria by diel period (sunrise, late morning, early afternoon, sunset).
C. Samples from plant A62.
D. Samples from plant A35.

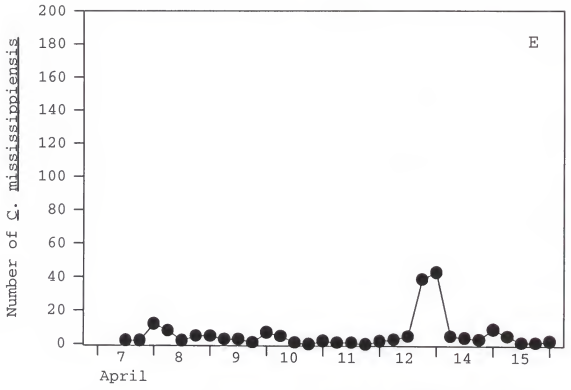


Figure 3-1. Number of Culicoides mississippiensis collected from Ilex vomitoria by diel period (sunrise, late morning, early afternoon, sunset). E. Samples from plant A129.

Table 3-4. Mean number of principal insects collected by diel period, spring 1995.

Diel Period	C. mississipp.	D. mutabilis	Thrips
1	19.2 AB*	29.4D	321.0AB
4	7.4 B	103.0B	201.6B
7	11.6 B	189.8C	307.4AB
10	28.0 A	279.2A	394.3A

*Means within columns followed by different letters are significantly different at the 0.05 probability level by REG-WQ multiple comparison test.

Table 3-5. Mean number of principal insects collected by plant, spring 1995.

Plant	C. mississipp.	D. mutabilis	Thrips
A36	29.8 A*	204.5B	59.4C
A14	24.0 A	287.3A	83.2C
A62	16.0 AB	93.9C	378.9B
A35	7.1 B	95.2C	334.8B
A129	5.8 B	58.2C	806.0A

*Means within columns followed by different letters are significantly different at the 0.05 probability level by REG-WQ multiple comparison test.

numbers in descending order correspond to the order in which plants were sampled during the flowering season. Significantly higher numbers of C. mississippiensis were collected from plants A36 and A14 than from A35 or A129 (ANOVA, $F=2.99$, $df=27,25$, $p<0.004$). The mean for A62 was intermediary to others, though not significantly different from the means for A36 and A14. More than 50 adult C. mississippiensis were collected on ten occasions from A36, on six from A14 and on five from A62, but only once from A35 and never from A14.

When data for all plants are combined, major collection peaks for C. mississippiensis can be identified every 3-4 days (Figure 3-2). The last such peak occurs on 4 April with two lesser peaks occurring on 8 and 12 April.

Examination of C. mississippiensis capture by bloom group, using the eight bloom groups defined in Chapter 2 (Table 2-5), showed that significant differences existed among bloom group means (ANOVA, $F=6.43$, $df=7,204$, $p<0.0001$). REG-WQ evaluation of bloom group means is given in Table 3-6. The mean for bloom group 5, corresponding to peak bloom and the start of bloom decline, was significantly greater than all other bloom group means except those of bloom groups 3 and 4.

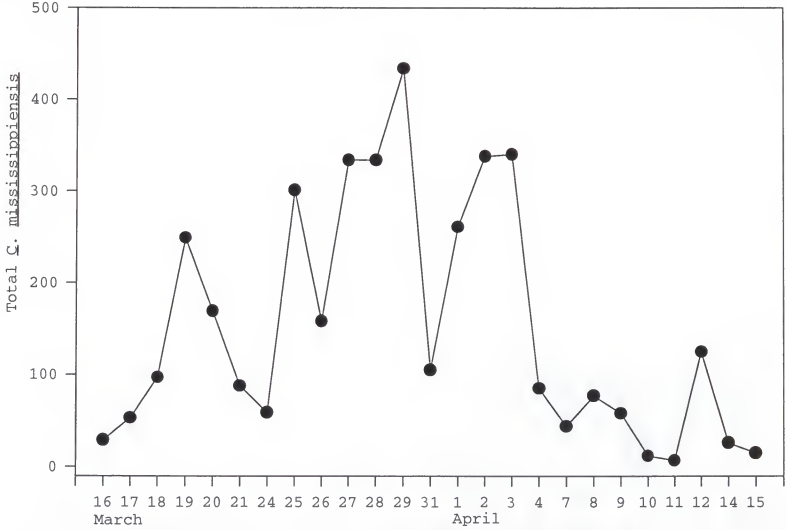


Figure 3-2. Daily totals for Culicoides mississippiensis collected from Ilex vomitoria, spring 1995.

Table 3-6. Mean number of principal insects collected per sample by bloom group, spring 1995. Bloom groups correspond to the indicated range of percent bloom.

Bloom group	N	C. mississip.	D. mutabilis	Thrips
1 (0-1%)	30	5.9 D*	67.5 CD	205.2 BC
2 (1-2%)	17	9.1 CD	129.9 BCD	415.8 ABC
3 (2-5%)	27	26.0 ABC	253.1 AB	201.6 BC
4 (5-22%)	28	28.1 AB	306.2 A	57.0 C
5 (22-5%)	18	37.3 A	203.0 ABC	123.3 C
6 (5-2%)	32	17.1 BCD	151.1 ABC	288.9 ABC
7 (2-1%)	27	11.6 BCD	100.1 BCD	590.9 A
8 (1-0%)	33	9.3 CD	31.5 D	521.4 AB

*Means within columns followed by different letters are significantly different at the 0.05 probability level by REG-WQ multiple comparison test.

Dasyhelea mutabilis. These ceratopogonids were the second most abundant insects in AS sweeper samples of flowering I. vomitoria in 1995. The combined totals of males and females collected for each plant by diel period are shown in graphs of Figure 3-3 A-E.

Daily rhythms. Combined data were analyzed by GLM and REG-WQ multiple comparison to identify diel foraging preferences. Mean (standard error) values for each diel period are shown in Table 3-4. Significant differences were found for all diel periods, and mean visitations increased from sunrise to sunset.

Seasonal rhythms. Combined data relative to source plants were analyzed by ANOVA and REG-WQ to identify seasonal visitation patterns. Plant samples represent early (A36, A14), middle (A62, A35) and late (A129) flowering season. Results, given in Table 3-5, show a significantly higher number of visitations made to early flowering plants than to middle and late flowering plants.

Peak numbers in samples are shown for 3-4 day intervals (Figure 3-4). A sharp decline in the number of D. mutabilis visiting I. vomitoria can be seen in the last third of the flowering season.

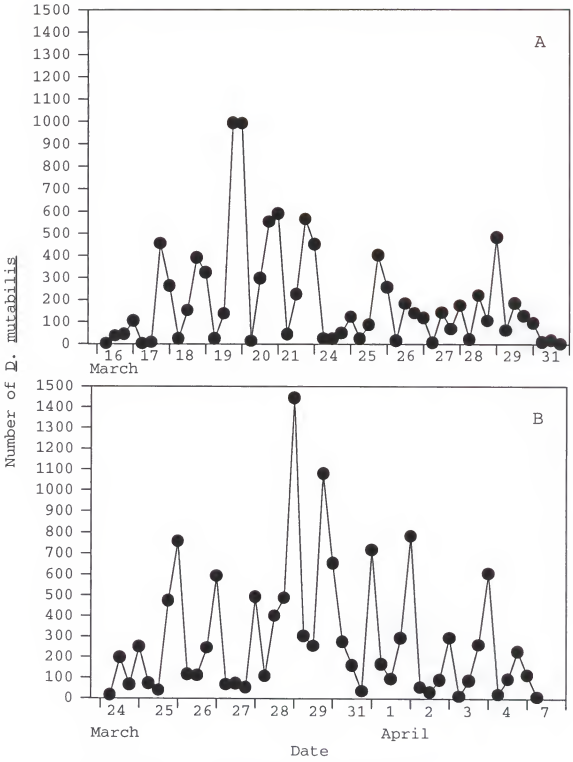


Figure 3-3. Number of D. mutabilis collected from I. vomitoria by diel period. (sunrise, late morning, early afternoon, sunset).
A. Samples from plant A36.
B. Samples from plant A14.

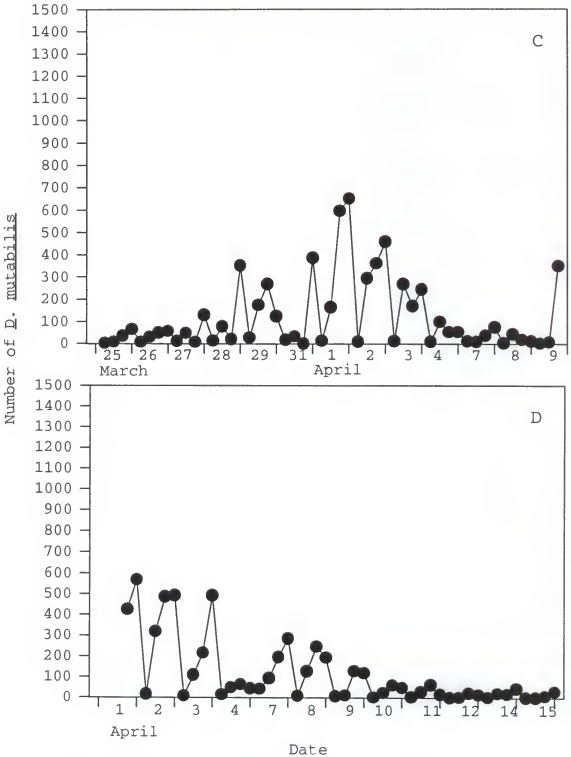


Figure 3-3. Number of D. mutabilis collected from I. vomitoria by diel period. (sunrise, late morning, early afternoon, sunset).
C. Samples from plant A62.
D. Samples from plant A35.

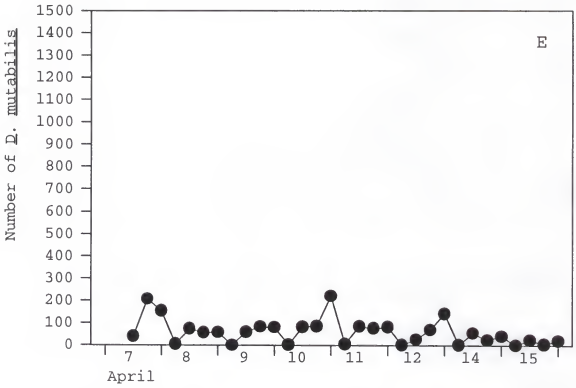


Figure 3-3. Number of D. mutabilis collected from I. vomitoria by diel period (sunrise, late morning, early afternoon, sunset). E. Samples from plant A129.

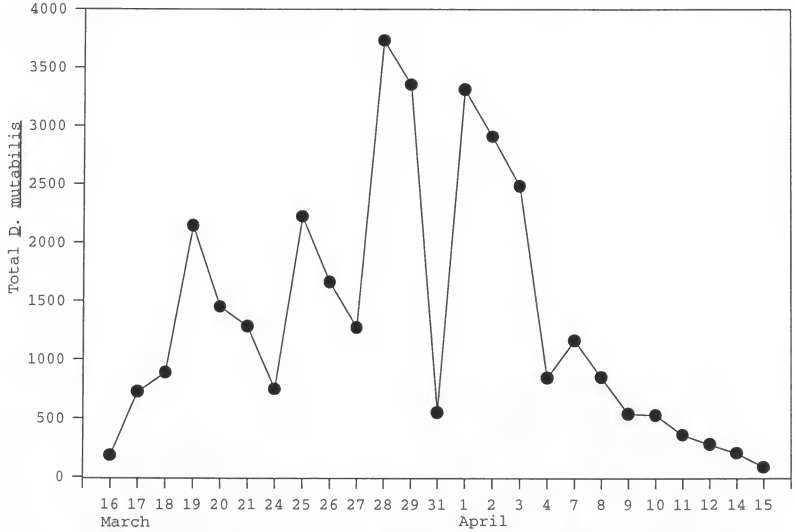


Figure 3-4. Daily totals for D. mutabilis collected from I. vomitoria, spring 1995.

Analysis of the mean number of D. mutabilis collected relative to the level of bloom of plants showed that D. mutabilis is attracted in higher numbers in bloom group 4, corresponding to late rise to peak bloom, followed by bloom groups 3, 5, and 6, in that order (ANOVA, $F=6.46$, $df=7,204$, $p<0.0001$). Mean capture for bloom group 4 was significantly greater than mean captures of bloom groups 1, 2, 7, and 8, corresponding to beginning and end stages of flowering. Results of REG-WQ evaluation of bloom group means is given in Table 3-6.

Thysanoptera. These were the most abundant insects in samples, though not evenly distributed throughout the flowering season. The majority of adult thrips were F. bispinosa. Many immatures were collected throughout the flowering season. More than 65,000 thrips were collected from flowering I. vomitoria, but no effort was made to distinguish the numbers of immatures from adults. The totals of thrips for each plant are represented in graphs of Figure 3-5 A-E.

Daily rhythms. Combined data was analyzed by GLM and REG-WQ multiple comparison to identify diel foraging preferences. Mean (standard error) values for each diel

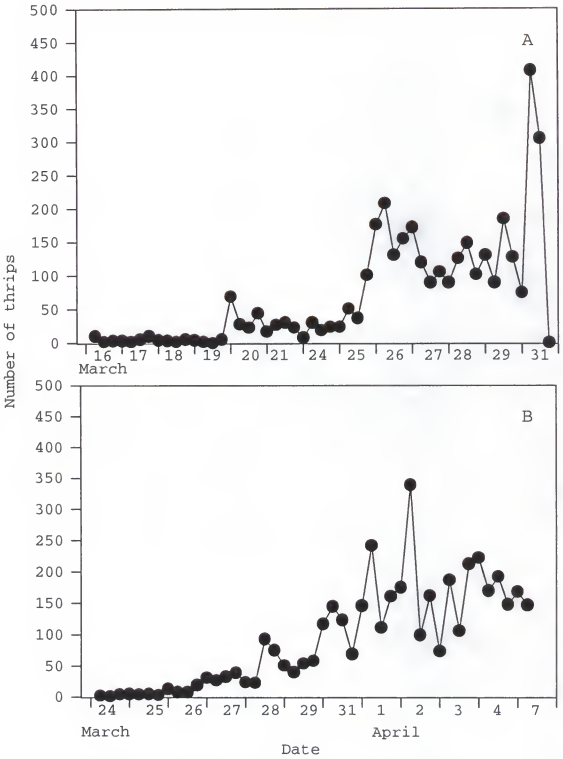


Figure 3-5. Number of thrips collected from I. vomitoria by diel period.
A. Samples from plant A36.
B. Samples from plant A14.

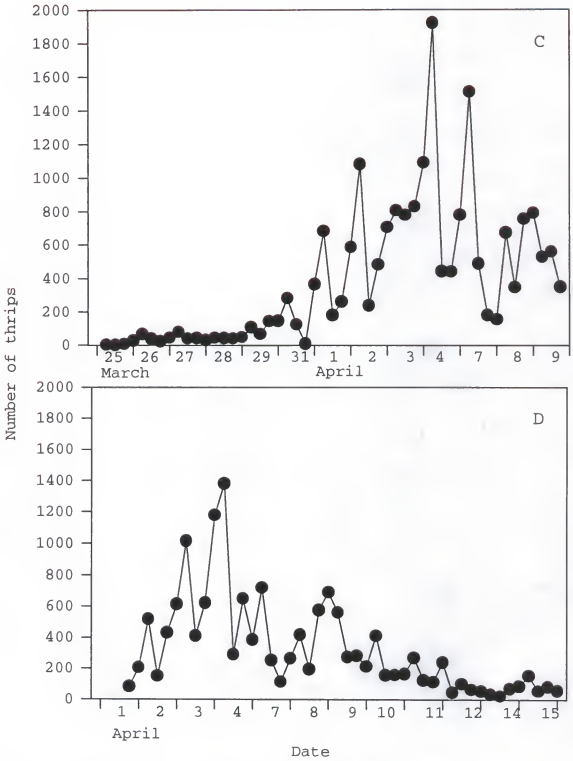


Figure 3-5. Number of thrips collected from I. vomitoria by diel period.
C. Samples from plant A62.
D. Samples from plant A35.

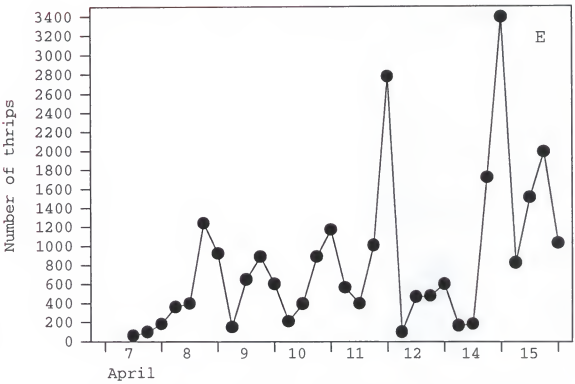


Figure 3-5. Number of thrips collected from I. vomitoria by diel period.
E. Samples from plant A129.

period are shown in Table 3-4. Relatively high numbers are collected in all diel periods, but the mean for diel period 10 is significantly higher than that of diel period 4.

Seasonal rhythms. Data analysis for thrips shows low population levels during the first two-thirds and a population explosion during the last third of the flowering season. Combined plant totals for the entire season are shown in Figure 3-6.

Examination of bloom group data for thrips showed highest mean numbers of thrips are associated with early and late phases of flowering, when percent bloom is at its lowest (ANOVA, $F=5.27$, $df=7,204$, $p<0.0001$.) Results of REG-WQ evaluation of bloom group data are given in Table 3-6. Graphs of bloom group data reflect a very different pattern of visitation for thrips to flowering I. vomitoria from those of the two major ceratopogonids (Figure 3-7).

Anthrone Tests

Positive anthrone tests were obtained for 427 of 768 (55.6%) midges tested. The number of male positives were 67 (53.2%), 23 (53.5%), 33 (55.0%), and 68 (43.9%) for diel periods 1,4, 7 and 10 respectively. Gravid female positives for the same periods were 57 (73.1%), 28 (65.1%), 51 (59.3%),

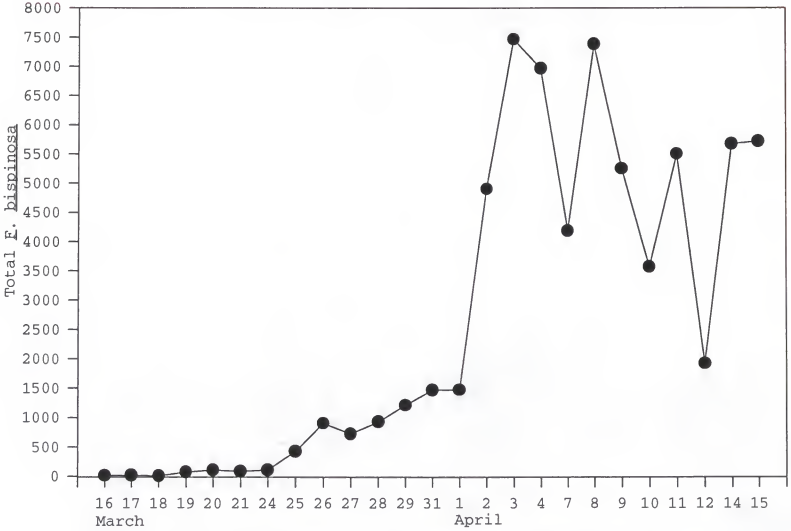


Figure 3-6. Daily totals for F. bispinosa collected from I. vomitoria, spring 1995.

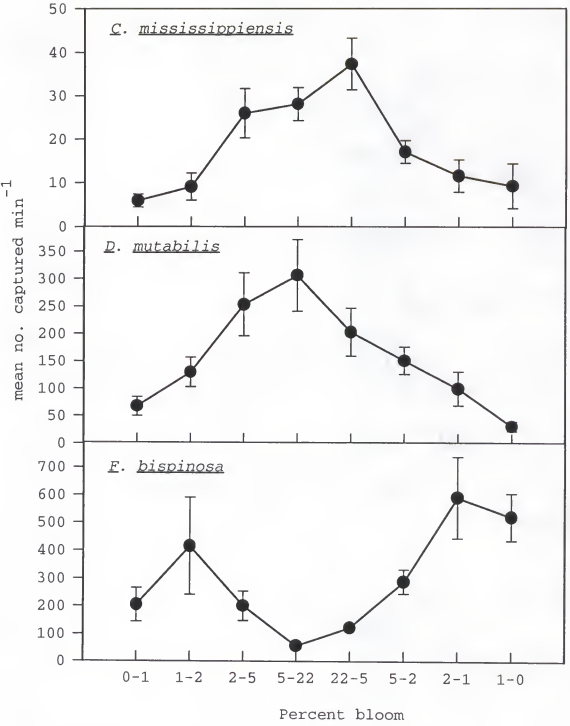


Figure 3-7. Mean 1 min vacuum samples for three insect species from flowering male I. vomitoria relative to percent bloom. Bloom increases to a maximum of 22% and then decreases to <1%.

and 100 (56.5%) (Fig. 3-8). An overall significant difference ($p \leq 0.10$) between male and gravid female anthrone positivity was found, with 236 of 384 (61.5%) females having sugar fed compared to 191 of 384 (49.7%) males. Male feeding rates were significantly higher during diel periods 1 ($X^2=14.426$, df=1), 4 ($X^2=7.688$, df=1), and 7 ($X^2=12.670$, df=1) than during diel period 10. Gravid female feeding rates decreased steadily from dawn to dusk, and those of periods 7 ($X^2=10.560$, df=1) and 10 ($X^2=19.735$, df=1) were significantly less than the rate for period 1.

Of 25 non-gravid, parous females collected and tested, 10 (40%) yielded positive anthrone results. The low number of parous females in samples indicates that the method of collection succeeded in excluding blood-meal seeking females. All controls were negative.

Biting rates of C. mississippiensis

Mean biting rates (range) were 140 (3-321), 79 (3-525), 117 (0-510), and 689 (51-2484) bites/h for diel periods 1, 4, 7 and 10, respectively. A comparison of mean biting rates and male/female floral samples for the four collection periods is shown in Figure 3-9. The biting rate for diel period 10 was significantly greater than rates for all other sample periods

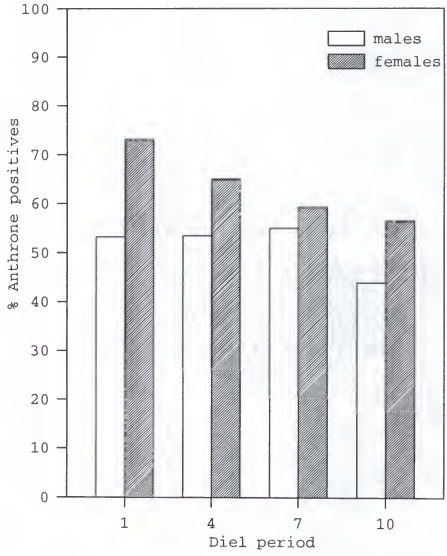


Figure 3-8. Percent sugar feeding of C. mississippiensis by diel period determined by the cold anthrone test.

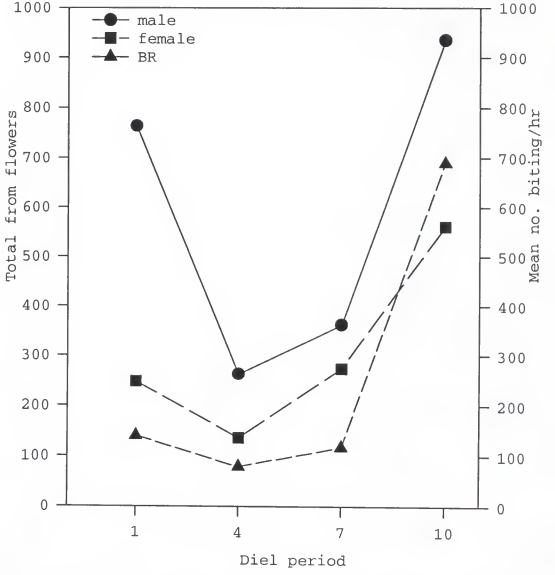


Figure 3-9. Comparison of the number of male and female C. mississippiensis collected from flowering male I. vomitoria with the mean number of midges biting per hour (BR) for corresponding diel periods.

and the rate for diel period 1 was significantly greater than that of diel period 4 (ANOVA, $F=23.11$, $df=3,92$, $p<0.001$). The lowest temperature for which biting activity was recorded was 10.5°C (8:25 A.M., 3 April) and the highest was 31°C (mid-afternoon, 9-11 April).

Discussion

Non-flowering Samples

Samples of non-flowering I. vomitoria taken in a study by Lillie and Kline (1986) contained very low numbers of C. mississippienisis compared to samples of flowering I. vomitoria. In the same study, higher numbers of C. mississippiensis were found in samples of non-flowering S. alterniflora, at emergence sites of C. mississippienisis, than non-flowering I. vomitoria, distant from emergence sites. The density of I. vomitoria is much lower in hammocks than is the density of S. alterniflora in the salt marsh. If C. mississippiensis had been attracted to non-flowering I. vomitoria then the numbers should have been at least as high as on S. alterniflora. Non-flowering I. vomitoria therefore seems to be an accidental resting site for C.

mississippiensis. No other arthropod taxa were reported by that study. Low numbers of all taxa reported by the present study indicate that non-flowering I. vomitoria serves as a resting site randomly accessed by C. mississippiensis and other insects. Higher numbers on flowering plants would therefore indicate attraction to the plants related to the flowers.

Flowering Season Samples

Uncommon visitors

Psocoptera These insects are incidental visitors, not intimately associated with flowering I. vomitoria.

Araneida. Spiders are non-nectar feeders, present on plants to feed on other arthropods that were feeding on the plants.

Homoptera. The yaupon psyllid, G. ilicis, is the only known gall-making psyllid frequenting yaupon holly (Mead 1983). Yaupon is the only plant on which this psyllid is known to breed. Numerous galls could be observed on male and female plants in the study site. Aphids were in such low numbers as to suggest these pests of citrus to be incidental visitors to yaupon holly. Immature whiteflies were neither present in samples nor on the undersurface of leaves,

suggesting that the few adults were accidental visitors to yaupon.

Lepidoptera. Since Geometrid adults are nocturnal feeders (Brantjes 1978), it is possible that adults visited yaupon to ingest nectar and to oviposit but were not present during the four diel collection periods.

Acari. AS sweep samples of flowering false-willow, Baccharis angustifolia Mich., also contained D. mutabilis parasitized by trombiculiids (unpublished personal data, October 1995). Trombiculiids were previously reported on D. mutabilis collected in California (Whitsel and Schoeppner 1967).

Diptera (Brachycera and Cyclorrhapha). Most of those flies were probably feeding on nectar, though no anthrone tests were carried out for confirmation.

Coleoptera. Beetles are commonly found on flowering plants feeding on pollen. It is such beetle transfer of pollen, incidental to feeding on the pollen, which Faegri and Pijl (1979) consider the most primitive form of pollination.

Hemiptera. Lygaeids are often thought of as seed eaters. However, given the lack of seeds on the male yaupon it is more likely that observed lygaeids were feeding either

on sap or other insects (Borror et al. 1992). The presence of immatures and adults suggests use of plants for breeding.

Collembola. Collembola are found primarily in leaf litter, but the data of this study indicate migration from litter to flowering vegetation in the early morning.

Common visitors

Hymenoptera. Observation of pollen on the bodies of larger Hymenoptera suggests they may have a role in pollination. Vespids may have been searching through foliage for Geometrid larvae or spiders. Higher numbers of chalcids visiting flowering yaupon in the afternoon suggest a preference to forage during the warmth of the afternoon.

Nematocera. It is likely that most nematocera visited yaupon flowers to obtain nectar.

Culicoides mississippiensis. Males are sugar feeders for their entire life span, but females are thought to feed on sugar only prior to producing the first batch of eggs and then convert to blood feeding. Males would therefore be expected to return to plants for successive sugar meals while females would be expected to make only one visit. Greater numbers of males than females in samples is in agreement with the results of samples collected by Lillie and Kline (1986).

Very common visitors

Dasyhelea mutabilis. These gnats are so small that they are able to obtain nectar from I. vomitoria flowers without making contact with anthers. What role they may play in pollination of other plants in the Gulf Coast region has not been evaluated. In Brazil Dasyhelea spp.are associated with flowering cocoa and may be cocoa pollinators (Winder 1977).

Although anthrone tests were not carried out on D. mutabilis specimens, it is very likely that they fed on nectar during visits to flowering I. vomitoria. They were commonly observed at nectar pools at the same time C. mississippiensis adults were feeding. Both males and females are known to feed only on sugars. A similar number of males and females in samples therefore confirms the expectation that individuals of both sexes would make return visits to plants for sugar feeding.

Male/female sample sex ratio was nearly 1:1, in contrast to the male-biased ratio of C. mississippiensis. Dasyhelea spp. are the only ceratopogonids whose females do not take blood meals.

Thysanoptera. Although considered primarily pollen feeders, thrips have been shown to feed on nectar, petals,

other flower parts and foliage of plants (Milne et al. 1996). In a study of thrips distribution and abundance in Florida, Childers et al. (1990) found that F. bispinosa made up 92% of all thrips collected from citrus and was the dominant thrips species on all non-citrus flowering plants sampled. Ilex vomitoria was not included in that study, but results of the present study indicate that it too is a major plant resource for this thrips species. It is the dominant species on flowering yaupon. The presence of great numbers of immatures as well as adults indicates use of flowering yaupon as a breeding site by F. bispinosa. For that reason I. vomitoria should be evaluated as a host plant that may be important to maintenance of F. bispinosa populations.

Visitation Rhythms

Culicoides mississippiensis

Daily rhythms. Preference for sugar feeding in crepuscular diel phases corresponds to crepuscular blood feeding preferences observed for this species and is discussed in more detail in the section on anthrone tests.

Seasonal rhythms. This species shows a bivoltine population pattern (Kline 1986), with peaks in spring and fall. Analysis of insect data for collections from flowering yaupon

provides a means for distinguishing important emergence phases within the overall spring population peak. Control based on attraction to floral volatiles aimed at biting midges at the time of expected emergence would most likely have greater impact on population reduction and be more cost effective than control applied indiscriminately throughout the spring.

Two possible explanations for observed collection peaks need to be considered. The first is that those peaks represent major emergence events of C. mississippiensis. This possibility could have been corroborated had emergence trap collections also been made during the study. The second is that peak attraction to flowers is occurring on those days. This possibility will be considered in Chapter 5 by statistical comparison of qualitative and quantitative floral volatile emissions on peak days with off-peak days.

Analysis of the mean number of C. mississippiensis collected relative to level of bloom of plants indicates that at least quantitative effects of floral volatile emissions are involved in increased insect attraction.

Dasyhelea mutabilis.

Daily rhythms. Dasyhelea mutabilis shows a marked preference for visiting flowering I. vomitoria in the late

afternoon and early evening. Comparatively fewer visitations were made in early morning.

Seasonal rhythms. Little is known of the population dynamics of D. mutabilis. Analysis of data for collections from flowering yaupon provides a window on spring emergence patterns for this species. Given the high numbers of D. mutabilis found on flowering I. vomitoria, it is likely that this species would be impacted by any control aimed at C. mississippiensis that is based on I. vomitoria floral attractants. Differences between target and non-target species relative to volatile attractants and foraging times may help in constructing a program that minimizes the impact on the non-target.

Seasonal population peaks show very close similarity in timing to those of C. mississippiensis. The decline in population during the last third of the flowering season was also similar to the decline observed for C. mississippiensis. Bloom group data for both D. mutabilis and C. mississippiensis clearly show attraction to I. vomitoria greatest when bloom is maximal and least when bloom is minimal. As was the case with C. mississippiensis, quantitative effects of floral volatile

production seem to be related to greater numbers of D. mutabilis visiting I. vomitoria.

Thysanoptera.

Daily rhythms. Constant populations of immatures are maintained on plants. Transitory peaks most likely represent recently arrived migrating swarms of adults. On one occasion the researcher, while standing over A129, observed such a swarm flying away from that plant.

Seasonal rhythms. The flowers of A62, A35 and A129 had heavy damage due to thrips, such as numerous unopened buds with holes eaten through petals. None of those three plants achieved the magnitude of bloom seen in A36 and A14. It is very likely that thrips infestations were responsible for those blooming deficiencies. Bloom group data suggest arrival of swarms on plants early in flowering, causing immediate reduction of bloom potential through damage to buds, and development of new migratory swarms during the plants' senescence. Low numbers of arthropods other than thrips in late season samples may reflect the impact of thrips injury to a plant's attraction for those arthropods. Attention should be given to the role played by I. vomitoria in maintenance and growth of F. bispinosa spring populations.

Anthrone Tests

Positive anthrone test results, in a range similar to those found for other autogenous nematocera (Bidlingmayer and Hem 1973, Magnarelli 1981, Reisen et al. 1986), are strong evidence of I. vomitoria nectar sugar ingestion by C. mississippiensis. During the study, no other plants bloomed as long and prolifically or were as widespread in hammocks as I. vomitoria. There were 145 I. vomitoria plants in the study site. Of the 131 that bloomed in spring 1995, 71 were male. Other plants in bloom during the study period included southern wax myrtle, Myrica cerifera L., black needlerush, Juncus roemerianus Scheele, and poison ivy, Toxicodendron radicans L. Only vacuum samples of poison ivy contained adult C. mississippiensis. Male I. vomitoria plants are shown by this study to be an important, widely available nectar source for both male and female C. mississippiensis during spring emergence when midge populations reach their highest levels.

A significant decrease in anthrone positives in the last sample period might be explained by reduced nectar availability. Increased competition with other insects, such as D. mutabilis and thrips species (90% of all insects in diel period 10), might have caused such temporal responses in

percent sugar feeding. However, diel period 10 samples with higher than mean anthrone positivity also had high numbers of D. mutabilis and thrips. Consequently, decreased feeding rates by midges cannot be explained by interspecific competition.

Decreased nectar production by flowers could also be reflected in lower number of anthrone positives during diel period 10. Nectar availability has been shown to vary seasonally, daily and diurnally (Kato 1993, Wright 1988), and may also relate to water availability (Petanidou and Voukou 1993). No data are available on the phenology of nectar production by I. vomitoria, nor were data collected during this study. It is therefore impossible to evaluate flower-based effects on nectar feeding.

Significant changes in nectar sugar feeding might best be explained as a function of biting midge foraging behavior. Lillie et al. (1988) found that the host-seeking behavior of C. mississippiensis peaked during periods 1 and 10 of the diel cycle. Biting rates for C. mississippiensis parous females found in this study were in agreement with the results of Lillie et al. (1988). Biting rate increases were consistently noted in diel period 10. Visitation of flowering I. vomitoria

by C. mississippiensis reflects similar periodicity, with highest numbers of midges collected from flowers during diel period 10 followed by period 1 (Figure 4-9). As increasing numbers of midges descend on I. vomitoria towards sunset, it would be expected that a greater proportion of samples would include recent arrivals that had not yet fed. This would be reflected by a lower frequency of anthrone positives for diel period 10. Higher feeding rates in earlier sample periods suggest that midges had been present and feeding for longer times than those collected in diel period 10.

The results of this study bear resemblance to those found by Van Handel and Day (1990) in a study of nectar feeding by Aedes taeniorhynchus (Wiedemann). In that study, low anthrone positive rates were found in Ae. taeniorhynchus collected before sunset and high rates for those collected at sunrise. It was determined that most sugar feeding took place soon after dark. If this were also the case with C. mississippiensis, high numbers of anthrone negatives would be expected before sunset. Biting midges collected in diel periods 4 and 7 would then be those that had fed during the night and rested on the plants through the following morning. However, this scenario seems unlikely due to the nature of

sampling. Vacuuming a plant at sunrise should effectively remove any insects which had lingered through the night. Insects collected must have arrived between vacuum events. It can therefore be concluded that C. mississippiensis feeding occurs throughout the day and possibly during the night.

The presence of sugar-fed parous females in collections indicates that females will seek nectar beyond the first oviposition event. Mullens (1985) suggested that higher sugar feeding rates in parous females than males and nulliparous females of C. variipennis reflects the need for energy to seek blood hosts. The same may be true for C. mississippiensis.

Biting rates.

The biting rates determined by this study confirmed the crepuscular host-seeking of C. mississippienisis reported by Lillie et al. (1988). This study further establishes crepuscular nectar-seeking by C. mississippienisis.

The significance of sugar feeding rates of midges collected directly from flowering plants is different from that of those collected from a blood-meal source. In the latter case males are absent from the sample and only parous females seeking blood to mature eggs are included. Flower-based collections can reveal behavior patterns for males,

nulliparous and parous females. It is likely that most males and females collected from flowers were there to feed on nectar. Negatives are those midges that had not yet ingested nectar at the time of collection, but may have tested positive if collected at some later time. Males may also use flowering I. vomitoria as a resting site more frequently than females, which disperse in search of blood meals following the first oviposition event. Little is known about the mating behavior of C. mississippiensis. Male swarms, over objects that contrast with the background, have been observed in Yankeetown (personal communications, D. Kline, P. Choate). Females may enter the swarms to mate. Although matings on flowering I. vomitoria were not observed during field collections for the present study, such behavior cannot be ruled out.

CHAPTER 4
FLORAL VOLATILE PHENOLOGY OF ILEX VOMITORIA

Introduction

The fragrance of flowers is a secondary attractant whose function is to draw potential pollinators to the plant to obtain a primary attractant, such as nectar (Faegri and Pijl 1979). In the process, many of those insects may coincidentally pick up pollen grains and transfer them to the stigma of flowers of that plant or its conspecifics. Most previous studies of floral volatiles relative to insect visitation have focused on euglossine bees (Cane and Tengö 1981, Whitten et al. 1988), fruit flies (Lewis et al. 1988), lepidoptera (Cantelo and Jacobson 1979, Haynes et al. 1991, Heath et al. 1992, Zhu et al. 1993) and weevils (Nielsen et al. 1995). Little has been done to identify chemicals from floral fragrance for the purposes of monitoring or control of medically important insects.

Fragrances that attract insects to plants, resulting in pollination, are synomones because the response produced upon perception of the fragrances is adaptively favorable to both insects and plants (Dicke and Sabelis 1988). The relationship between I. vomitoria and C. mississippiensis may not be that of a synomone induced partnership. Direct observation of insects taking nectar from male flowers during the study revealed that it is possible for C. mississippiensis adults to approach the nectar pool without ever contacting the anthers, perched relatively high above the pool. Although accidental pollination by biting midges may occur, it is unlikely that yaupon pollination depends on these or other equally small ceratopogonids. Much of yaupon pollination probably results from visits by larger Diptera and Hymenoptera.

Nevertheless, C. mississippiensis is an important visitor to yaupon, depending on the flowers as a major source of nectar. It is tempting to conclude that yaupon floral fragrance is kairomonal in the case of C. mississippiensis since it would appear that only the insects benefit from their attraction to the flowers (Dicke and Sabelis 1988).

Floral fragrance usually results from emission of many different chemicals, often referred to as essential oils,

blended in different amounts. Most essential oils are produced in the cytoplasm of the epidermis and mesophyll cells of sepals or osmophore and diffuse to the air at favorable temperatures (Fahn 1979). Those not emitted may enter lipid biosynthesis pathways in roots and rhizomes or be oxidized for energy (Croteau 1988). Essential oils include terpenes, phenylpropanoids, alcohols, aldehydes, esters, acids and sulfur compounds (Metcalf and Metcalf 1992). The main fragrance of flowers results from the presence of terpenes (Fahn 1979), having high volatility due to low molecular weights.

Terpenes are synthesized via the mevalonic acid pathway. Isoprene units, with five carbon atoms, form the structural base of terpenes. The most important terpenes of floral fragrance are monoterpenes (two isoprene units) and sesquiterpenes (three isoprene units). Oxidation of terpenes by plants yields alcohols, aldehydes, ketones and esters (Metcalf and Metcalf 1992). A tremendous variety of essential oils has been reported and is summarized by Knudsen et al. (1993).

Fragrance specifically associated with pollen grains is also thought to be a potential secondary attractant. In a study of the chemicals found in pollenkitt, the oily coating

of angiosperm pollen, Dobson (1988) established a wide range of diversity of essential oils found on the pollen of 69 different plants. Such diversity points to species specific pollen-related olfactory cues for arthropod visitors to flowering plants. Ilex vomitoria produces copious amounts of pollen, and so the essential oils associated with a pollen plume should also be evaluated as potential attractants.

Headspace extraction has been used extensively in analysis of floral volatiles (Heath et al. 1992, Patt et al. 1988, Tatsuka et al. 1990) and for preparation of extracts for use in bioassays (Healy and Jepson 1988). The method is described in detail by Heath et al. (1992). The process involves collection of volatiles from flowers onto an absorbent, usually a porous polymer, by creating a flow of air across the flowers and then through the absorbent. The method allows for extraction of very volatile compounds that are often missed by other methods (Budde and Eichelberger 1979).

Choice of absorbent can be critical. Tenax has been indicated as a poor absorbent for carbonyl compounds (Tatsuka et al. 1990) and pinene (Patt et al. 1988). Super Q is the absorbent of choice, accommodating those not collected by Tenax as well as most other floral volatiles. Because of

artifact production associated with solvent extraction and steam distillation, Knudsen et al. (1993) endorse headspace extraction as the preferred method for work with floral volatiles. A further advantage of headspace extraction over other extraction techniques is the ability to extract volatiles as they are produced by living flowers.

In the identification of floral essential oils, a major consideration is whether flowers to be sampled are attached to plants or detached. Flowering shrubs and trees are subjected to highly variable conditions of humidity, temperature and lighting. Working with detached flowers theoretically allows for the study of intrinsic volatile emissions free of the influence of climate. Studies have been done in which only detached flowers were sampled (Pellmyr et al. 1987, Tatsuka et al. 1990), but the actual value of such an approach can only be evaluated in comparative studies. Loughrin et al. (1993) found no difference in volatile production by attached and detached flowers of Nicotiana suaveolens Lehman sampled over 24 hours. When extended diurnal rhythmicity is of interest, detached flowers may not always be useful. In a study carried out over four days, Matile and Altenburger (1988) took volatile samples from both detached and attached flowers of

several different plants at 3 hour intervals. Their results show that while some plants maintain normal rhythmic fragrance production, whether detached or not, others lose diurnal rhythmicity when detached. Heath et al. (1992) also showed that release rates of chemicals may be altered in detached flowers. In that study, benzyl acetate increased significantly in detached flowers of night-blooming jessamine while phenyl acetaldehyde decreased significantly. Mookherjee et al. (1986) also found important differences between the chemicals emitted by intact versus excised flowers of jasmine, lilac and Easter lily plants.

Gas chromatography has provided a window on the chemical world in which we and the organisms we study live. In entomology its uses are varied, including studies on the mechanisms of intraspecific recognition (Bonavita-Cougourdan 1987), mate selection (Peschke 1987), strategies for host selection for parasitoids (Phelan et al. 1991) and taxonomic relationships (Carlson and Service 1980, Wirth and Morris 1985). Gas chromatography enables the quantitative characterization of the components of a mixture extracted from some source. The process depends on the differential dissolving of those components by the stationary phase of the

column (Perry 1991). GC may also be combined with mass spectrometry to provide a means of qualitative identification of the components of a mixture. On passing from GC into the mass spectrometer, molecules are ionized and then separated according to their mass-to-charge ratios, producing a spectrum that can be matched by computer to a library of such spectra (Clement and Karasek 1985).

The objectives of the present study were to collect and identify volatiles of intact flowering male I. vomitoria four times a day throughout the flowering season. Data was statistically analyzed to identify phenological patterns of essential oil production by plants and correlation of those patterns with C. mississippiensis visitations. Collection of pollen was also made to determine how much pollen contributes to floral fragrance.

Materials and Methods

Volatile Collection

The sampling apparatus consisted of a Reynolds® oven bag (turkey size, 62 cm x 48 cm, made of nylon resin, exposure temperatures not to exceed 204°C) pulled over a segment of

flowering branch and supported by a two ring tomato cage (distal diameter 24 cm, proximal diameter 18 cm). The cage support was held onto the branch by duct tape, and the oven bag tightly tied around the branch with string. Each oven bag had two pipette ports reinforced externally by duct tape and centrally positioned on opposite sides of the bag, one placed at the closed end and the other halfway towards the open end. A Y-shaped wooden support was used to prevent the sampling apparatus from bending to the ground.

Volatiles were collected by removal of air from the oven bag, using an air vacuum pump with DC motor connected to a 12 volt deep cycle marine battery, across a Pasteur pipette containing 4 mg Super Q (Alltech, Deerfield, IL). The absorbent was sandwiched by glass wool, that had been washed in pentane, inside the pipette. Ambient air drawn into the oven bag was filtered through activated charcoal (20-60 mesh)(Sigma) held by glass wool inside a second pipette. Pipettes were inserted into the two pipette ports and held in place by two overlapping strips of duct tape. An air flow of 250 ml/min was monitored with an in-line air flow meter.

A blank sample and sample of non-flowering vegetation was made prior to the start of the flowering season. Once

flowering had started, one-hour samples were collected, four times a day. Differences between internal oven bag temperatures and the ambient air temperature were monitored by an indoor-outdoor thermometer. Shading of the oven bag by a campers foil emergency blanket was provided when needed and effectively maintained internal temperatures at or below that of the ambient temperature.

On completion of volatile sampling, pipettes were wrapped in aluminum foil, placed in a jar and held on ice until return to Gainesville, where they were transferred to an ultra low (-70°C) freezer. Each oven bag and air filter pipette was used only once a day and heated at 95°C for one hour before reuse. Pollen was collected from intact flowers on Whatman No. 1 filter paper attached to the exhaust outlet of an AFS backpack sweeper. It was then wrapped in aluminum foil and handled in the same way as volatile samples.

Samples were prepared by extraction with methylene chloride. Only the first ten drops were collected, yielding a volume of approximately 30 µl solution. Nearly all of the extract can be found in the first ten drops of solvent passed through the adsorbent (C. M. Whitten, personal communication). To this was added 20 µl internal standard in solution

containing 100 ng standard/µl. Each 1 µl of the final mixture injected into the GC therefore contained 40 ng of the internal standard. The internal standard used was tridecane, a 13 carbon alkane. Selection of tridecane was made following injection of a 1 µl mixture of C_9-C_{16} alkanes (prepared by the laboratory of Mr. R. Heath, USDA/CMAVE) with an extracted sample of volatiles from I. vomitoria. Since the C_{13} alkane showed no overlap, yielding a distinctly separate peak from those of I. vomitoria volatiles, it was chosen for use as the internal standard.

Methylene chloride was used for extraction of pollenkitt following the method of Dobson (1988). Approximately 1 mg of pollen was washed in 0.5 ml methylene chloride for 20 seconds and filtered through Whatman No. 1 filter paper fitted to a syringe. The short solvent extraction period allows for removal of pollenkitt, located in the exine, without removing other chemicals found in the intine of the pollen (Dobson et al. 1988). To 30 µl pollenkitt extract was added 20 µl tridecane as a standard for quantification of identified compounds. Two pollenkitt samples were processed by GC/MS as previously described.

GC/MS Analysis

Access to GC/MS was limited. However, GC without coupled mass spectroscopy was available for the duration of the project. It was therefore decided to identify I. vomitoria volatiles by running selected samples on GC/MS and then, using the same protocol, extrapolate those results to chromatograms produced by GC alone for the majority of samples. Volatile identification was made on pollen and selected whole flower samples by a HP 5995A gas chromatograph/mass spectrometer with data analysis by Teknivent Vector/Two data analysis system. All other samples were processed for phenological data on a HP 5890 Series II Gas Chromatograph following the same GC protocol which had been used on the HP 5995A GC/MS. Hydrogen was used as the carrier gas at a flow rate of 7.7 ml min^{-1} in a fused silica capillary column of intermediate polarity (SPB-5, 30m x 0.25mm i.d., 0.25µm film). Injection was splitless, on-column using a HP Automatic Sampler 7673A and detection by Flame Ionization Detector (FID). The GC was temperature programmed from 60°C to 290°C at 3°C min^{-1}, injector set at 63°C and detector at 340°C. Blank samples, containing solvent mixed with standard, were run at the start of each series of samples.

Peaks were matched by their relative distance from the retention time of the internal standard as well as their relative distance from each other. Mean retention times were calculated from weighted retention times for peaks found in two or more plants. Peaks recorded in blanks were produced by solvent or impurities in solvent and were deleted from each plant's GC profile.

Data Analysis

In order to identify phenological changes in volatile production all data obtained by GC was converted to mass of chemical per microliter extract for a one hour sample (ng/µl-hr). To do this, a correction factor was first applied to each peak area to compensate for variation caused by procedure. The correction factor, the ratio of the peak area to peak area of the internal standard, is multiplied by the mass of the standard to give the mass of the chemical under consideration (Dean 1995). Because of the large number of samples included in this project, division of each internal standard area by the sample's mean internal standard area was carried out. This was then used as the correction factor in the above mass calculation. The mass thereby obtained, after multiplication by 40ng (mass of internal standard per µl),

was the mass of that particular chemical produced by the entire cluster of flowers sampled on an individual plant for one hour. A sample calculation is given in Appendix C.

Data sets for each identified chemical were examined by ANOVA and REG-WQ in order to determine diel and seasonal patterns of emission. Combined data sets for chemicals were matched to percent bloom values and C. mississippiensis capture data. Analysis was made by Canonical Correlation and Regression (SAS Institute, 1989). The goal of Canonical Correlation Analysis was to identify the combination of chemicals (identified by GC/MS) most strongly correlated with high numbers of biting midges in AFS sweep samples. Capture values for C. mississippiensis were transformed with square roots and analysis performed on the transformed values. Regression in reference to Canonical Correlation Analysis was used to develop an ANOVA model based on chemical data most strongly correlated with high capture of C. mississippiensis. This process involved regression tests that evaluated the impact of removal of selected sets of chemicals. The model was refitted to include only those chemicals which were significant ($p<0.05$) or marginally significant($0.05<p<0.10$). Similar treatment was made on D. mutabilis data.

Results

Sample Analysis

Blank and non-flowering vegetation samples

Blank and non-flowering vegetation volatile samples were both negative for identifiable peaks.

Pollenkitt samples

The majority of peaks produced by GC/MS analysis of pollenkitt samples were found in a range of very high retention times. Chemicals that eluted at retention times below 40 minutes, the range of *I. vomitoria* floral volatiles, are listed in Table 4-1. Most are either aldehydes or acids. Those chemicals comprised less than 4 % by peak area of all pollenkitt volatiles. A sample GC trace of pollenkitt volatiles is included in Appendix C.

Floral samples

A total of 194 volatile samples were analyzed by GC/MS. A summary of peak retention times for chemicals found in two or more plants is given in Table 4-2. A sample GC trace of floral volatiles is shown in Appendix C. A total of 51 chemicals eluted, of which 20 were identified by GC/MS. Identified chemicals include (in order of elution) dimethyl

Table 4-1. Essential oils identified by GC/MS from pollen of *Ilex vomitoria*, spring 1995. Only chemicals that eluted in a range of retention times corresponding to that for floral volatiles are listed.

Retention Time	Chemical
5.33	unidentified
6.50	unidentified
7.78t	heptanal
13.45	2-octenal
14.57	heptanoic acid
15.50	nonanal
20.20	decanal
22.70	2-decenal
23.32	nonanoic acid
33.38	pentadecane

Table 4-2. Essential oils of flowering male *I. vomitoria* identified by GC/MS. Mean retention times are based on the number of samples (N) in which a chemical was found.

Mean RT	N	Chemical
3.021	97	
3.076	105	
3.492	98	
3.823*	53	dimethyl benzene
4.467	15	
4.672	14	
4.930	10	
5.393	3	
5.562	6	
5.678	11	
5.758	3	
6.245	3	
6.631*	140	α-pinene
7.896*	104	β-pinene
8.967*	34	(2-methyl propyl) benzene
9.617*	22	limonene
9.905*	98	cis-β-ocimene
10.346*	145	trans-β-ocimene
10.412*	55	1,2-diethyl benzene
10.626*	141	1,3-diethyl benzene
10.846*	144	1,4-diethyl benzene
11.712	4	
11.844*	21	linalool oxide
12.317	35	linalool

t= tentative identifications *= confirmed identifications

Table 4-2 (continued)

Mean RT	N	Chemical
12.461*	35	
13.041*	135	4,8-dimethyl-1,3,7-nonatriene
13.648	2	
13.844*	56	phenyl acetonitrile
14.918	27	
15.259t	147	ethyl benzaldehyde
15.590	16	
15.901t	153	ethenyl benzaldehyde
16.451	12	
16.876t	49	dodecenal
17.666	9	
17.998	9	
18.727	94	
19.451*	161	1-(2,4-dimethyl phenyl) ethanone
20.282*	157	1-(4-ethyl phenyl) ethanone
20.817	18	
22.492	9	
23.864	12	
24.220	9	
24.401	3	
25.656	15	
26.890	71	
27.527	5	
28.261	10	
29.116	35	
29.930	12	
30.257*	76	E,E-α-farnesene

benzene, α-pinene, β-pinene, (2-methyl propyl) benzene, limonene, cis-β-ocimene, trans-β-ocimene, 1,2-diethyl benzene, 1,3-diethyl benzene, 1,4-diethyl benzene, linalool oxide, linalool, 4,8-dimethyl-1,3,7-nonatriene, phenyl acetonitrile, ethyl benzaldehyde, ethenyl benzaldehyde, dodecenal, 1-(2,4-dimethyl phenyl) ethanone, 1-(4-ethyl phenyl) ethanone, and E,E-α-farnesene. The GC program was unable to consistently discriminate between trans-β-ocimene and 1,2-diethyl benzene. The latter peak always appeared as a shoulder on the trans-β-ocimene peak. In 23 samples from plants A14, A62 and A129 discrimination between the two was sufficient for independent integration of their peak areas.

The mass totals of all identified chemicals by diel period are 157.4 ng/µl-hr, 234.8 ng/µl-hr, 241.4 ng/µl-hr, and 230.3 ng/µl-hr for diel periods 1, 4, 7, and 10, respectively. Since each hour's volatile sample was extracted to 30 µl and mixed with 20 µl internal standard/solvent mixture, each µl contains 1/50 th of the total hourly sample. The mean hourly samples by diel period for the 20 identified chemicals are therefore 7.87 µg, 11.74 µg, 12.07 µg and 11.52 µg for diel periods 1, 4, 7 and 10, respectively. Data for individual chemicals are listed in Table 4-3.

Table 4-3. Mean mass of each essential oil identified by GC/MS from flowering male Ilex vomitoria spring 1995.

Chemical	Mean mass (ng)/µl-hr for diel: 1	4	7	10
dimethyl benzene	0.7	1.4	1.5	1.7
α-pinene	4.4A*	9.1BC	11.7C	7.6BC
β-pinene	1.4A	3.6B	5.4C	3.1B
(2-methyl propyl) benz.	0.2A	0.3AB	0.3A	0.8B
limonene	0.2	0.4	0.9	0.3
cis-β-ocimene	0.8A	2.9B	4.1C	2.2B
t.-β-oc./1,2-diet.benz.	82.8	127.0	113.7	113.9
1,3-diethyl benzene	22.3	25.6	27.2	32.0
1,4-diethyl benzene	5.7	6.9	7.7	8.6
linalool oxide	0.4	1.6	1.7	0.9
linalool	0.2	0.6	0.7	1.0
4,8-dimet.-1,3,7-nonat.	1.4A	4.9B	9.4C	5.2B
phenyl acetonitrile	0.5	5.8	8.1	3.2
ethyl benzaldehyde	3.1A	3.9AB	4.3B	5.0B
ethenyl benzaldehyde	6.5	7.2	8.5	9.3
dodecenal	0.1A	1.1B	1.8B	1.2B
1-(2,4-dimet.phen.)eth.	16.1	19.1	21.5	24.0
1-(4-eth.phen.)eth.	8.2	9.5	9.3	10.8
E,E-α-farnesene	2.4	3.9	5.1	1.2

*Means within rows followed by different letters are significantly different at the 0.05 probability level by REG-WQ multiple comparison test.

Data Analysis

Phenology of volatile emissions

In the case of all identified chemicals, emissions increase from diel 1 to diel 7. The patterns for 1(4-ethyl phenyl) ethanone and combined trans-β-ocimene/1,2-diethyl benzene appear to differ, but there are no significant differences for their diel emission masses. The increase from diel 1 to diel 4 is statistically significant in α-pinene, β-pinene, cis-β-ocimene, 4,8-dimethyl-1,3,7-nonatriene and dodecenal (ANOVA, $P<0.001$, REG-WQ).

Two general patterns of volatile production from diel 7 to diel 10 can be seen in the data. Reduction of volatile production from diel 7 to diel 10 can be seen in about half of all identified chemicals. Included in this group are α-pinene, β-pinene, limonene, cis-β-ocimene, linalool oxide, 4,8-dimethyl-1,3,7-nonatriene, phenyl acetonitrile, dodecenal and E,E-α-farnesene. Of those, reduction from diel 7 to diel 10 is statistically significant in β-pinene (ANOVA, $F=5.80$, $df=81,122$, $p<0.0001$), cis-β-ocimene (ANOVA, $F=6.30$, $df=81,122$, $p<0.0001$), 4,8-dimethyl-1,3,7-nonatriene (ANOVA, $F=3.69$, $df=81,122$, $p<0.0001$).

The second pattern seen in 8 of the remaining chemicals is that of continued increase in emissions from diel 7 to diel 10. Significant increases were found only for (2-methyl propyl) benzene.

Cannonical correlation analysis

Culicoides mississippiensis. Analysis of all chemical data obtained in the study by Canonical Correlation produced a correlation of 0.493. Standardized canonical coefficients (W1) for each chemical are listed in Table 4-4. High captures of C. mississippiensis are correlated to high amounts of chemicals with positive coefficients and low amounts of chemicals with negative coefficients. The results show that high amounts of ethyl benzaldehyde and α-pinene combined with low amounts of 1,4-diethyl benzene are most strongly correlated with high captures of C. mississippiensis. These coefficients can be used to direct selection of chemicals to be removed from consideration in regression analysis.

When bloom groups are compared by REG-WQ relative to W1, bloom group 5 is shown to have a significantly higher mean W1 value than all other bloom groups. Bloom group 5 is followed by bloom groups 4, 6, and 3 in that order. These results confirm that the highest correlation between volatile

Table 4-4. Standardized canonical coefficients of indicated chemicals for correlation with the square root of capture of C. mississippiensis. High amounts of chemicals with positive coefficients and low amounts of chemicals with negative coefficients correlate to high square root of capture.

Chemical	W1
dimethyl benzene	-0.5015
α-pinene	1.3847
β-pinene	-0.6179
(2-methyl propyl) benzene	0.0699
limonene	-0.2563
cis-β-ocimene	0.0814
t.-β-ocimene/1,2-diethyl benzene	-0.3269
1,3-diethyl benzene	0.1856
1,4-diethyl benzene	-2.3726
linalool oxide	-0.0780
linalool	0.0025
4,8-dimethyl-1,3,7-nonatriene	-0.4755
phenyl acetonitrile	-0.1490
ethyl benzaldehyde	1.4101
ethenyl benzaldehyde	0.7241
dodecenal	0.2890
1-(2,4-dimethyl phenyl) ethanone	0.5979
1-(4-ethyl phenyl) ethanone	-0.3292
E,E-α-farnesene	0.2140

composition and highest captures of C. mississippiensis also correlate to the periods of peak flowering of I. vomitoria. A similar examination of W1 values relative to diel periods shows highest mean W1 for diel 10 followed by 1, 4 and 7 in that order. However, none of the diel periods were significantly different from others. Analysis of variance for all chemicals yields an F value of 3.502 and $p<0.0001$, showing that at least one of the chemicals is a significant predictor for the response variable ($capture^{1/2}$). The model has an r^2 value of 0.2656, indicating that other factors not considered in the analysis play an important role in predicting the response variable. A list of p values for each individual chemical included in the initial model is shown in Table 4-5.

Test statements were used in regression analysis to identify chemicals that could be dropped from the model without significantly affecting the model. For example, when 1,4-diethyl benzene and dimethyl benzene are evaluated in a regression test, the p value for the group is 0.0001, showing that they need to be kept in the model. On the other hand, a test of the group linalool oxide, linalool and E,E-α-farnesene produces a p value of 0.9975, indicating that the group can be

Table 4-5. Probability values of indicated chemicals for inclusion in a model predicting high square root of capture of C. mississippiensis.

Chemical	p
intercept	0.0001
dimethyl benzene	0.0620
α-pinene	0.0003
β-pinene	0.0228
(2-methyl propyl) benzene	0.6630
limonene	0.2969
cis-β-ocimene	0.7701
t.-β-ocimene/1,2-diethyl benzene	0.2263
1,3-diethyl benzene	0.7681
1,4-diethyl benzene	0.0183
linalool oxide	0.8722
linalool	0.9915
4,8-dimethyl-1,3,7-nonatriene	0.0334
phenyl acetonitrile	0.7156
ethyl benzaldehyde	0.0139
ethenyl benzaldehyde	0.4826
dodecenal	0.3544
1-(2,4-dimethyl phenyl) ethanone	0.4622
1-(4-ethyl phenyl) ethanone	0.4468
E,E-α-farnesene	0.4734

Table 4-6. Probability values of indicated chemicals for inclusion in a model predicting high square root of capture of C. mississippiensis following regression analysis.

Chemical	p	Parameter Estimate
intercept	0.0001	3.170
dimethyl benzene	0.0101	-0.269
α-pinene	0.0001	0.098
β-pinene	0.0584*	-0.105
1,4-diethyl benzene	0.0001	-0.431
4,8-dimethyl-1,3,7-nonatriene	0.0354	-0.069
ethyl benzaldehyde	0.0029	0.484
ethenyl benzaldehyde	0.0390	0.207

* Indicates marginal significance

dropped without significantly reducing the fit of the regression model.

After running numerous tests of chemical groups, based on low absolute W1 values, a model for predicting optimal capture of C. mississippiensis was developed including α-pinene, β-pinene, 1,4-diethyl benzene, dimethyl benzene, 4,8-dimethyl-1,3,7-nonatriene, ethyl benzaldehyde and ethenyl benzaldehyde. P values and parameter estimates, used in model construction, are shown in Table 4-6. The model based on those parameter estimates is:

$$\text{capture}^{1/2} = 3.17 - 0.269\{\text{dimethyl benzene}\} + 0.098\{\alpha\text{-pinene}\} - 0.104\{\beta\text{-pinene}\} - 0.431\{\text{1,4-diethyl benzene}\} - 0.069\{\text{4,8-dimethyl-1,3,7-nonatriene}\} + 0.484\{\text{ethyl benzaldehyde}\} + 0.207\{\text{ethenyl benzaldehyde}\}$$

Plots of residuals versus predictors were used to look for higher order polynomial trends, but none were apparent.

Dasyhelea mutabilis. Capture data for D. mutabilis was handled in the same way for canonical correlation analysis as was the data for C. mississippienisis. This analysis produced a correlation of 0.530. Standardized canonical coefficients

(W1) for each chemical are listed in Table 4-7. Analysis of variance for all chemicals yields an F value of 3.781 and $p<0.0001$ (df=19,184), showing that at least one of the chemicals is a significant predictor for the response variable ($capture^{1/2}$). The model has an r^2 value of 0.2808, indicating that other factors not considered in the analysis play an important role in predicting the response variable. A list of p values for each individual chemical included in the initial model is shown in Table 4-8.

Essential oils most highly correlated with high $capture^{1/2}$ of D. mutabilis included α-pinene, 1,4-diethyl benzene, linalool oxide, 1-(2,4-dimethyl phenyl) ethanone, ethyl benzaldehyde, dimethyl benzene, and phenyl acetonitrile. Probability values and parameter estimates for those essential oils following removal of other chemicals from the model are shown in Table 4-9. Based on those values a model may be developed of the form:

$$capture^{1/2} = 8.025 + 0.228\ \{\alpha\text{-pinene}\} - 1.097\ \{1,4\text{-diethyl benzene}\} - 2.288\{\text{linalool oxide}\} + 0.199\{1\text{-}(2,4\text{-dimethyl phenyl) ethanone}\} + 1.306\{\text{ethyl benzaldehyde}\} - 0.856\{\text{dimethyl benzene}\} + 0.298\{\text{phenyl acetonitrile}\}$$

Plots of residuals versus predictors did not reveal higher order polynomial trends to be included in the model.

Table 4-7. Standardized canonical coefficients of indicated chemicals for correlation with with the square root of capture of D. mutabilis. High amounts of chemicals with positive coefficients and low amounts of chemicals with negative coefficients correlate to high square root of capture.

Chemical	W1
dimethyl benzene	-0.4567
α-pinene	1.1385
β-pinene	-0.2020
(2-methyl propyl) benzene	0.2072
limonene	-0.4611
cis-β-ocimene	0.4575
t.-β-ocimene/1,2-diethyl benzene	-0.4564
1,3-diethyl benzene	0.2590
1,4-diethyl benzene	-1.6480
linalool oxide	-1.0172
linalool	-0.0619
4,8-dimethyl-1,3,7-nonatriene	-0.0753
phenyl acetonitrile	-0.6844
ethyl benzaldehyde	0.9143
ethenyl benzaldehyde	0.5333
dodecenal	0.3862
1-(2,4-dimethyl phenyl) ethanone	1.9740
1-(4-ethyl phenyl) ethanone	-0.8788
E,E-α-farnesene	-0.1695

Table 4-8. Probability values of indicated chemicals for inclusion in a model predicting high square root of capture of D. mutabilis.

Chemical	p
intercept	0.0001
dimethyl benzene	0.0607
α-pinene	0.0009
β-pinene	0.4083
(2-methyl propyl) benzene	0.1549
limonene	0.0392
cis-β-ocimene	0.0710
t.-β-ocimene/1,2-diethyl benzene	0.0630
1,3-diethyl benzene	0.6497
1,4-diethyl benzene	0.0702
linalool oxide	0.0216
linalool	0.7731
4,8-dimethyl-1,3,7-nonatriene	0.7085
phenyl acetonitrile	0.0659
ethyl benzaldehyde	0.0772
ethenyl benzaldehyde	0.5680
dodecenal	0.1726
1-(2,4-dimethyl phenyl) ethanone	0.0079
1-(4-ethyl phenyl) ethanone	0.0259
E,E-α-farnesene	0.5308

Table 4-9. Probability values of indicated chemicals for inclusion in a model predicting high square root of capture of D. mutabilis following regression analysis. Parameter estimates are used for construction of the model.

Chemical	p	Parameter Estimate
intercept	0.0001	8.025
α-pinene	0.0001	0.228
1,4-diethyl benzene	0.0001	-1.097
linalool oxide	0.0006	-2.288
1-(2,4-dimethyl phenyl) eth.	0.0149	0.199
ethyl benzaldehyde	0.0140	1.306
dimethyl benzene	0.0038	-0.856
phenyl acetonitrile	0.0102	0.298

Discussion

Sample Analysis

Blank and Non-flowering samples

The lack of volatiles from blank and non-flowering samples indicates that the method of volatile sampling was not able to detect chemicals from either the oven bag or duct tape used for seals. Absence of detectable quantities of volatiles may have been due to the relatively short time of sample collection. In the case of non-flowering vegetation, lack of volatiles may also relate to the fact that all vegetation was from the previous season's growth. As noted in Chapter 3, new leaves are produced after flowering has occurred.

Pollenkitt samples

High retention times of pollenkitt peaks indicate that those chemicals have low volatility (W. M. Whitten, personal communication). None of the pollenkitt volatiles with retention times less than 40 minutes was also found in floral volatiles. Pollenkitt volatile contributions to overall floral fragrance were therefore not detectable by the protocol of this study. In a study done on two Rosa species, Dobson et

al. (1987) also found that the volatiles of pollenkitt were distinctly different from those of flowers. This does not mean that pollenkitt volatiles are necessarily specific to pollenkitt. Heptanal, octanal, nonanal, heptanoic acid and nonanoic acid, identified from pollenkitt in this study, have also been identified from flowers of the chestnut, Castanea creata (Yamaguchi and Shibamoto 1980). Heptanal, decanal, and nonanal have been identified in the floral fragrance of orchids (Omata et al. 1990, Borg-Karlson and Groth 1986).

Flowering season samples

The absence of data for a chemical from any plant does not necessarily mean that the chemical was not emitted by the plant since emission rates may have been below the sensitivity threshold for the procedure. Many of the floral essential oils identified in this study are commonly found in emissions of other flowering plants (Chang et al. 1988, Nielsen et al. 1995, Patt et al. 1988, Pellmyr et al. 1987). Significant electroantennogram response has been identified to limonene by the European grapevine moth (Gabel et al. 1992) and to limonene, linalool and both cis- and trans-β-ocimene by alfalfa seed chalcids. Both limonene and α-pinene have been identified as attractants for scolytid beetles and boll

weevils and linalool as an attractant for silkworms (Berenbaum and Siegler 1992). Results such as these showing insect attraction to essential oils common to many flowering plants supports future investigation of possible attraction by C. mississippiensis to those chemicals.

Data Analysis

Phenology of volatile emissions

Diffusion of terpenes through plant cell walls and cuticle is a temperature dependent event (Fahn 1979). The GC data of the present study support field observation of increased floral scent in late morning and early afternoon when higher temperatures would favor essential oil diffusion to the plant exterior.

Quantities of monoterpene emissions by flowers have also been shown to vary relative to the age of plants and to time within the flowering season (Chang et al. 1988). Other studies have shown that some floral terpenes are released rhythmically while others are not (Matile and Altenburger 1988, Nielsen et al. 1995). Only 1(4-ethyl phenyl) ethanone and combined trans-β-ocimene/1,2-diethyl data did not fit one of the two rhythmic emission patterns shown for other I. vomitoria essential oils. Since there were no significant

differences shown for any of their diel period emissions, they may also fit one of the two emission patterns.

Cannonical correlation analysis

Culicoides mississippiensis.

Occasionally attractants for insects are single compounds, such as methyl eugenol for tephritids (Fraenkel 1959). However, synergistic interaction of essential oils in attraction systems is a more common phenomenon. Guerin et al. (1983) determined that carrot flies are more strongly attracted to a mixture of hexenal and trans asarone than to either of the chemicals alone. A study on the attraction of sunflower volatiles to the red sunflower seed weevil showed that a mixture of five chemicals produced attaction, but that removal of only one chemical from the blend reduced attraction (Roseland et al. 1992).

The model for C. mississippiensis capture predicts the response of capture number to a change in the output of any one chemical. For example, if the release of α-pinene was increased by one unit, and all other chemicals held constant, the capture$^{1/2}$ of C. mississippiensis trapped would be expected to increase by a factor of 0.098. If on the other hand, β-pinene output increased by one unit while all other chemicals

were held constant, the capture$^{1/2}$ of C. mississippiensis trapped would be expected to decrease by a factor of 0.104. The model does not necessarily predict response to simultaneous changes in more than one chemical. The results indicate that potential attractants for C. mississippiensis could include from one to all of the chemicals in the model. Chemical blends, in which levels of chemicals not highly correlated with capture of D. mutabilis are manipulated, would be of special interest.

The results for D. mutabilis indicate that it may be possible to tailor a volatile blend that is attractant to C. mississippiensis but not to D. mutabilis. Test blends should avoid manipulation of α-pinene, 1,4-diethyl benzene, ethyl benzaldehyde, and dimethyl benzene and focus on chemicals not shared by the two models.

CHAPTER 6
SUMMARY AND CONCLUSIONS

Research Results

It is unrealistic to expect to find quick solutions to an insect control problem as challenging as that of Culicoides mississippiensis Hoffman. The goal of the present study was to evaluate the association between this pest and flowering yaupon holly. The general outcomes of the research were an increased understanding of that association and several leads for future study in the field of electrophysiology. Specific results are summarized for the plant, the plant's feeding guild, and floral volatiles.

Yaupon Holly

The yaupon holly, Ilex vomitoria Aiton, was intensively studied during its entire flowering season from 16 March to 15 April 1995 on a hammock in Yankeetown, Florida. All plant positions were plotted on a map of the hammock and data collected for sex ratio, size and start of flowering. Percent bloom as a function of time was determined for five male

plants based on an estimate of flowering potential and actual flowering data.

Sex ratio

A total of 145 plants were mapped, of which 71 were male, 59 female, and 15 of undetermined sex. The sex ratio of 1.20:1 (male:female)for flowering plants is comparable to sex ratios for other hollies (Cavigelli et al. 1986, Richards 1988).

Size

Of the 71 male plants, 26 (36.6%) were small, 25 (35.2%) were medium and 20 (28.2%) were large. Female size totals were 26 small (44.1%), 14 were medium (23.7%) and 19 were large (32.3%). All but one plant of undetermined sex were small.

Start of flowering

Plants started flowering over a 33 day span. The mean start day for males (day 11) was significantly different from that of females (day 18). All small plants begin flowering significantly later than other plants. Medium male plants began flowering significantly later than large male plants, but there was no significant difference between medium and

large female plants. There was significant interaction between the start of flowering and mean daily temperature.

Percent bloom

Five large male plants were included in a study of the visiting insect community and floral volatile emissions over the entire flowering season. Male plants were chosen for the study because they had significantly more buds per cm stem (20.4) than female plants (2.9) and would serve as better sources of insects and volatiles.

Plants that started flowering early in the season reached significantly higher percent bloom than plants that began flowering late in the season. Mean peak bloom ranged from 1.9% (late season plant) to 18.5% (early season plant). Depression of flowering in late season plants is significantly associated with high thrips populations. Thrips damage to buds was common in late season plants and was most likely the cause of reduced flowering. Eight percent bloom classes were constructed to facilitate correlation of insect and volatile samples to flowering.

Yaupon Feeding Guild

One-minute vacuum samples of arthropods were collected by AFS sweeper before the start of the flowering season and four

times daily throughout the flowering season. Pre-flowering means for important insect taxa were less than means for taxa collected from flowering plants. Insect data for flowering season samples provided a profile of arthropod visitations.

Profile of arthropod visitors

More than 90% of all arthropods were either thrips (59.5%) or the non-biting ceratopogonid Dasyhelea mutabilis Kieffer (30.6%). Common visitors to flowering yaupon were C. mississippiensis (3.4%), other nematocera (2.2%) and chalcidoid Hymenoptera (1.7%). Uncommon visitors were Collembola (0.7%), Hemiptera (0.6%), Coleoptera (0.3%), other Diptera (0.3%), Acari (0.2%), Lepidoptera (0.1%), Homoptera (0.1%), Araneida (0.1%), Pscoptera (<0.1%) and Mollusca (<0.1%).

Visitation rhythms

Dasyhelea mutabilis. These insects had similar dynamics to C. mississippiensis relative to their visits of flowering yaupon. Highest numbers were found in late afternoon, in the first half of the flowering season, and were significantly higher at periods of peak bloom. However, the number of visitations was significantly lower in the early morning than at any other time of the day.

Thrips. Thrips visitations to flowering yaupon were highest during the second half of the flowering season. Their numbers were high throughout the day, but highest in the late afternoon. Their numbers were also significantly higher at periods of low bloom, during the initiation of flowering and flowering senescence.

Culicoides mississippiensis. Preference for visiting flowering yaupon in crepuscular periods was shown, with the mean number of these biting midges collected before sunset significantly higher than any other time of the day except the early morning. Peak visitations occurred every three to four days in the first half of the flowering season (16 March to 4 April) dropping to lower levels after that time. Visitations by C. mississippiensis were significantly higher at periods of peak bloom. Anthrone tests were carried out on a subsample of C. mississippiensis to identify nectar feeding behavior. Nectar feeding was compared to blood-meal seeking behavior.

Sugar and blood feeding behavior

Positive anthrone tests were obtained for 55.6% of biting midges tested, indicating that C. mississippiensis is attracted to flowering yaupon to feed on nectar. There were significantly more female anthrone positives (61.5%) than male

anthrone positives (49.7%). Males and females took nectar meals throughout the day. Lowest positive rates for both male and female were found for early evening possibly due to the high number of recent arrivals on plants.

During the study period, the biting rate for female C. mississippiensis in the early evening (689 bites/h) was significantly greater than at any other time of the day, followed by early morning (140 bites/h). The range of biting rates over the study period was 0-2484 bites/h. Host seeking was recorded over a temperature range of 10.5°C-31.0°C.

Floral Volatiles

Samples of blank, non-floral vegetation and pollen samples were analyzed by GC/MS to make comparison with floral volatiles. No identifiable peaks were found in either blank or non-floral vegetation samples. Pollen samples contained mostly high molecular weight (low volatility) compounds, with small amounts of heptanal and nonanal. Pollen was not shown to account for any of the chemicals present in floral samples.

Identification

More than 190 volatile samples were analyzed for chemical composition. Of 51 chemicals which eluted, 20 were at high enough levels to be identified. They were dimethyl benzene,

α-pinene, β-pinene, (2-methyl propyl benzene, limonene, cis-β-ocimene, trans-β-ocimene, 1,2-diethyl benzene, 1,3-diethyl benzene, 1,4-diethyl benzene, linalool oxide, linalool, 4,8-dimethyl-1,3,7-nonatriene, phenyl acetonitrile, ethyl benzaldehyde, ethenyl benzaldehyde, dodecenal, 1-(2,4-dimethyl phenyl) ethanone, 1-(4-ethyl phenyl) ethanone, and E,E-α-farnesene. Mean production of these chemicals was in a range of 7.87 µg/hr (early morning) to 12.07 µg/hr (early afternoon). Microliter volumes of samples were analyzed by GC/MS and mean mass/µl-hr ranged from 0.1 ng (dodecenal, early morning) to 127.0 ng (combined trans-β-ocimene and 1,2-diethyl benzene).

Phenology

Two patterns of volatile emission were detected. Emission of nine chemicals increased from early morning to early afternoon and then decreased to sunset. Of those the decrease from afternoon to sunset is significant for β-pinene, cis-β-ocimene, and 4,8-dimethyl-1,3,7-nonatriene. The second pattern is that of increase from early morning up to sunset. Eight chemicals show this pattern, including dimethyl benzene, (2-methyl propyl) benzene, 1,3-diethyl benzene, 1,4-diethyl benzene, linalool, ethyl benzaldehyde, ethenyl benzaldehyde

and 1-(2,4-dimethyl phenyl) ethanone. Only (2-methyl propyl) benzene showed a significant increase from early afternoon to sunset.

Canonical correlation analysis

Data for all identified chemicals was analyzed by canonical correlation, yielding a correlation of 0.493. Standardized canonical correlation coefficients were used to direct selection of chemicals to be included in regression analysis for development of a linear model for optimal capture of C. mississippiensis. This analysis showed that seven of the twenty chemicals are significantly correlated to peak biting midge visitation of flowering yaupon. Those seven are dimethyl benzene, α-pinene, β-pinene, 1,4-diethyl benzene, 4,8-dimethyl-1,3,7-nonatriene, ethyl benzaldehyde and ethenyl benzaldehyde.

Seven essential oils of I. vomitoria are significantly correlated to visitation of D. mutabilis to flowering male plants. Those chemicals are α-pinene,1,4-diethyl benzene, linalool oxide, 1-(2,4-dimethyl phenyl) ethanone, ethyl benzaldehyde, dimethyl benzene and phenyl acetonitrile. These results indicate that D. mutabilis may be attracted to a different blend composition than C. mississippiensis.

Conclusions

The results of the research indicate that C. mississippiensis visits flowering I. vomitoria to obtain nectar. Canonical correlation identified seven essential oils emitted by male I. vomitoria flowers as possible secondary attractants for C. mississippiensis. Seven essential oils were also correlated to high capture of D. mutabilis. Four essential oils were highly correlated for both C. mississippiensis and D. mutabilis, but the remaining three were different. Yuval (1992) recommends studies to identify attractants that would concentrate mosquitoes at bait stations. The results of this study provide leads for formulating essential oil blends that may be capable of concentrating C. mississippiensis at bait stations.

Although it is possible that other factors, such as flower color and weather conditions, play important roles in attracting biting midges to flowering yaupon, those seven chemicals merit further investigation. A behavioral bioassay should first be developed. The Y-tube model developed by Blackwell et al. (1993) should be considered for this purpose. Studies have shown attractant responses to 1-octen-3-ol by

other species of biting midges (Kline et al. 1994, Ritchie et al. 1994). Evaluation of the attractancy of C. mississippiensis to 1-octen-3-ol should be made to determine its suitability for use in development of a Y-tube bioassay. Electroantennogram trials, following the method of Blackwell et al. (1993) should then be carried out, first on whole flower extract, then on discrete chemicals and blends. Comparison of the models for C. mississippiensis and D. mutabilis developed by canonical correlation analysis should guide selection of chemicals for candidate attractant blends. Discrete chemicals or blends that produce significant EAG responses should next be tested for possible attraction by a working bioassay.

Any attractants for C. mississippiensis may also prove to be attractants for D. mutabilis, thrips and a variety of other arthropods. Timed release of attractants in a trapping system could prevent capture of non-targets. Release only in the early morning would both target C. mississippiensis at one of its most active times and avoid peak activity of Hymenoptera and D. mutabilis, the most likely non-target victims of control based on yaupon volatiles. Nevertheless, susceptibility of D. mutabilis to yaupon-based control must be

thoroughly evaluated to prevent undesired reductions of its population. Such evaluation should also determine the feasibility of late afternoon release of attractants in order to target C. mississippiensis when it is most active.

APPENDIX A

FLORAL PHENOLOGY OF ILEX VOMITORIA

The following table contains data relative to the start of flowering and size of each of the Ilex vomitoria plants included in the dissertation study. Plants are coded with the letter A, corresponding to the study site hammock, and number. All plant locations are indicated on the map shown in figure 3-5. Sex determination was made on the basis of flower structure. Plants that did not flower were considered to be of undetermined sex. Size was approximated visually as small, medium or large. Start of flowering was made by direct observation of plants every two to three days.

Table A-1. Floral phenology of Ilex vomitoria on Hammock A in Yankeetown, Florida. Sex is listed as female (F), male (M), or undetermined (U). Sizes are small (S), medium (M), or large (L). Plants indicated by * are those included in dissertation sampling exercises.

ID	Sex	Size	Start Bloom
A1	F	M	3/29
A2	F	L	3/24
A3	F	S	NONE
A4	M	S	3/24
A5	U	S	NONE
A6	U	S	NONE
A7	F	S	3/29
A8	M	S	3/24
A9	M	L	3/24
A10	M	L	3/21
A11	F	S	3/29
A12	M	L	3/21
A13	M	M	3/18
A14*	M	L	3/21
A15	F	S	3/29
A16	M	L	3/18
A17	F	M	4/9
A18	M	L	3/24
A19	M	L	3/24
A20	M	L	3/24
A21	F	L	3/27
A22	F	L	4/9
A23	M	M	4/3
A24	F	S	3/29
A25	M	M	3/27
A26	U	S	NONE
A27	F	S	3/18
A28	F	L	3/24
A29	M	S	3/24
A30	M	M	3/18

Table A-1. Continued

ID	Sex	Size	Start Bloom
A31	F	S	3/29
A32	M	L	3/29
A33	F	S	3/24
A34	F	L	4/7
A35*	M	L	3/24
A36*	M	L	3/14
A37	F	L	3/18
A38	M	S	4/7
A39	M	L	3/16
A40	M	L	3/18
A41	F	S	3/29
A42	F	S	NONE
A43	F	S	3/31
A44	F	S	4/3
A45	F	S	3/31
A46	F	L	3/29
A47	M	S	3/21
A48	M	S	3/24
A49	M	S	3/21
A50	F	L	3/29
A51	F	S	3/29
A52	U	L	NONE
A53	M	L	3/18
A54	F	L	3/24
A55	U	S	NONE
A56	F	L	3/31
A57	F	L	3/29
A58	F	L	3/27
A59	M	M	3/27
A60	F	S	3/31
A61	F	S	4/7
A62*	M	L	3/24
A63	F	L	4/7
A64	F	S	4/7
A65	F	L	3/29

Table A-1. Continued

ID	Sex	Size	Start Bloom
A66	M	M	3/16
A67	M	M	3/21
A68	U	S	NONE
A69	M	S	3/27
A70	U	S	NONE
A71	F	S	4/3
A72	M	S	4/7
A73	F	S	3/31
A74	F	S	4/3
A75	F	M	3/31
A76	F	S	4/12
A77	M	M	3/27
A78	M	M	3/24
A79	U	S	NONE
A80	U	S	NONE
A81	F	M	4/3
A82	F	M	4/3
A83	U	S	NONE
A84	M	M	3/24
A85	F	M	4/3
A86*	F	L	3/31
A87	M	M	3/24
A88	F	M	3/21
A89	F	M	3/24
A90	F	M	3/31
A91	F	M	3/24
A92	M	S	3/24
A93	M	S	3/21
A94	F	S	4/7
A95	M	S	3/24
A96	M	S	3/29
A97	F	S	3/24
A98	M	S	3/21
A99	M	S	3/27
A100	M	M	3/24

Table A-1. Continued

ID	Sex	Size	Start Bloom
A101	M	S	3/24
A102	U	S	NONE
A103	U	S	NONE
A104	F	S	4/15
A105	F	M	4/3
A106	M	M	3/24
A107	F	M	3/27
A108	M	L	3/24
A109	M	M	3/27
A110	M	M	3/27
A111	F	M	3/29
A112	M	M	3/24
A113	M	S	3/24
A114	M	S	3/27
A115	M	S	3/24
A116	M	S	3/27
A117	F	S	4/7
A118	F	S	4/7
A119	M	M	3/24
A120	M	L	3/21
A121	M	M	3/24
A122	M	L	3/21
A123	M	M	3/24
A124	M	M	3/21
A125	F	S	4/7
A126	F	S	4/7
A127	M	M	3/27
A128	M	S	3/31
A129	M	L	3/29
A130	F	M	3/29
A131*	F	L	3/31
A132	M	S	3/24
A133	M	S	3/29
A134	F	S	4/7
A135	U	S	NONE

Table A-1. Continued

ID	Sex	Size	Start Bloom
A136	F	S	3/27
A137	M	S	3/27
A138	F	S	3/24
A139	M	L	3/24
A140	M	S	3/24
A141	M	M	3/24
A142	M	M	3/24
A143	M	M	3/27
A144	M	S	4/7
A145	M	M	3/29

APPENDIX B
PERCENT BLOOM DATA

The following tables contain data relative to the percent bloom of each plant included in the volatile study. Diel periods correspond to hour-long sample periods at sunrise, late morning, early afternoon and sunset. Percent bloom was determined by multiplying the total sample segment length (used in volatile sampling) by the average number of buds/cm (males: 20 buds/cm, females: 3 buds/cm) to yield a flower potential value (the total number of flowers that the sample segment could produce.) The number of flowers on the sample segment during any diel period may then be divided by the flower potential to give percent bloom.

Sample Calculation (A36 during diel period 1 on 3/16/95):

Total Sample Segment Length = 106.5 cm

$$\text{Mean buds/cm} = \underline{\text{x 20}}$$
$$\text{Flower potential} = 2130$$

$$\text{Percent Bloom} = \frac{\text{No. open flowers}}{\text{Flower potential}} = \frac{3}{2130} = 0.1\%$$

Table B-1. Percent bloom data for plant A36. Total sample segment length of 106.5 cm. Flower potential of 2130. Numbers shown in each diel period are for the number of open flowers and percent bloom indicated in parentheses.

	Diel Period			
Date	1	4	7	10
3/16	3(0.1)	6(0.3)	4(0.2)	4(0.2)
3/17	2(0.1)	5(0.2)	6(0.3)	5(0.2)
3/18	6(0.3)	28(1.3)	30(1.4)	30(1.4)
3/19	54(2.5)	67(3.1)	70(3.3)	73(3.4)
3/20	62(2.9)	89(4.2)	123(6.2)	128(6.0)
3/21	163(7.7)	160(7.5)	169(7.9)	169(7.9)
3/24	414(19.4)	390(18.3)	382(17.9)	380(17.8)
3/25	464(21.8)	405(19.0)	374(17.6)	365(17.1)
3/26	409(19.6)	380(17.8)	376(17.7)	370(17.4)
3/27	436(20.5)	386(18.1)	344(16.2)	340(16.0)
3/28	369(17.3)	340(16.0)	294(13.8)	294(13.8)
3/29	143(6.7)	125(5.9)	109(5.1)	109(5.1)
3/31	104(4.9)	104(4.9)	104(4.9)	-

Note: No data collection on 3/31, diel period 10 due to inclement weather.

Table B-2. Percent bloom data for plant A14. Total sample segment length of 148.0 cm. Flower potential of 2960. Numbers shown in each diel period are for the number of open flowers and percent bloom indicated in parentheses.

	Diel Period			
Date	1	4	7	10
3/24	16(0.5)	29(1.0)	30(1.0)	29(1.0)
3/25	70(2.4)	70(2.4)	79(2.7)	77(2.6)
3/26	130(4.4)	131(4.4)	129(4.4)	125(4.2)
3/27	157(5.3)	155(5.2)	149(5.0)	147(5.0)
3/28	243(8.2)	240(8.1)	239(8.1)	239(8.1)
3/29	246(8.3)	240(8.1)	220(7.4)	220(7.4)
3/31	446(15.1)	446(15.1)	446(15.1)	-
4/1	-	-	362(12.2)	362(12.2)
4/2	81(2.7)	100(3.4)	106(3.6)	105(3.5)
4/3	94(3.2)	92(3.1)	90(3.0)	90(3.0)
4/4	99(3.3)	75(2.5)	70(2.4)	70(2.4)
4/7	4(0.2)	-	-	-

Note: No data collection on 3/31, diel period 10 and 4/1, diel periods 1 and 4, due to inclement weather. No data collection after diel period 1 on 4/7 due to end of flowering.

Table B-3. Percent bloom data for plant A62. Total sample segment length of 182.0 cm. Flower potential of 3640. Numbers shown in each diel period are for the number of open flowers and percent bloom indicated in parentheses.

	Diel Period			
Date	1	4	7	10
3/25	25(0.7)	28(0.8)	34(0.9)	28(0.8)
3/26	34(0.9)	28(0.8)	28(0.8)	26(0.7)
3/27	44(1.2)	46(1.3)	46(1.3)	45(1.2)
3/28	172(4.7)	150(4.1)	152(4.2)	152(4.2)
3/29	146(4.0)	95(2.6)	88(2.4)	88(2.4)
3/31	59(1.6)	59(1.6)	59(1.6)	-
4/1	-	-	44(1.2)	44(1.2)
4/2	7(0.2)	30(0.8)	35(1.0)	35(1.0)
4/3	32(0.9)	35(1.0)	35(1.0)	35(1.0)
4/4	68(1.9)	40(1.1)	33(0.9)	33(0.9)
4/7	6(0.2)	13(0.4)	13(0.4)	13(0.4)
4/8	6(0.2)	6(0.2)	6(0.2)	6(0.2)
4/9	6(0.2)	6(0.2)	6(0.2)	6(0.2)

Note: No data collection on 3/31, diel period 10 and 4/1, diel periods 1 and 4, due to inclement weather.

Table B-4. Percent bloom data for plant A35. Total sample segment length of 213.0 cm. Flower potential of 4260. Numbers shown in each diel period are for the number of open flowers and percent bloom indicated in parentheses.

	Diel Period			
Date	1	4	7	10
4/1	-	-	64(1.5)	64(1.5)
4/2	126(3.0)	135(3.2)	149(3.5)	149(3.5)
4/3	151(3.5)	110(2.6)	110(2.6)	110(2.6)
4/4	73(1.7)	45(1.1)	31(0.7)	31(0.7)
4/7	119(2.8)	159(3.7)	159(3.7)	159(3.7)
4/8	170(4.0)	126(3.0)	125(2.9)	125(2.9)
4/9	126(3.0)	96(2.2)	96(2.2)	96(2.2)
4/10	73(1.7)	60(1.4)	60(1.4)	60(1.4)
4/11	51(1.2)	45(1.1)	35(0.8)	31(0.7)
4/12	60(1.4)	51(1.2)	42(1.0)	42(1.0)
4/14	33(0.8)	33(0.8)	33(0.8)	33(0.8)
4/15	18(0.4)	18(0.4)	12(0.3)	7(0.2)

Note: No data collection on 4/1, diel periods 1 and 4, due to inclement weather.

Table B-5. Percent bloom data for plant A129. Total sample segment length of 181.0 cm. Flower potential of 3620. Numbers shown in each diel period are for the number of open flowers and percent bloom indicated in parentheses.

	Diel Period			
Date	1	4	7	10
4/7	-	33(0.9)	33(0.9)	33(0.9)
4/8	30(0.8)	29(0.8)	29(0.8)	29(0.8)
4/9	34(0.9)	20(0.6)	20(0.6)	20(0.6)
4/10	39(1.1)	49(1.4)	49(1.4)	49(1.4)
4/11	91(2.5)	70(1.9)	58(1.6)	53(1.5)
4/12	101(2.8)	83(2.3)	81(2.2)	81(2.2)
4/14	53(1.5)	53(1.5)	53(1.5)	53(1.5)
4/15	25(0.7)	17(0.5)	7(0.2)	8(0.2)

Note: No data collection on 4/7, diel period 1 due to unexpected end of bloom by A14 as indicated by 4/7, diel period 1 sample of that plant and replacement by A129.

APPENDIX C
SAMPLE GAS CHROMATOGRAPHY TRACES

Sample gas chromatography traces of pollen and floral volatile samples analyzed by the protocol described in Chapter 4 are included in the following two pages. The floral volatile sample is that of diel period 2 on 31 March 1995 for plant A36. The tridecane internal standard peak is found at 21.2 min on each GC trace. Peaks below 3 minutes were produced by the solvent.

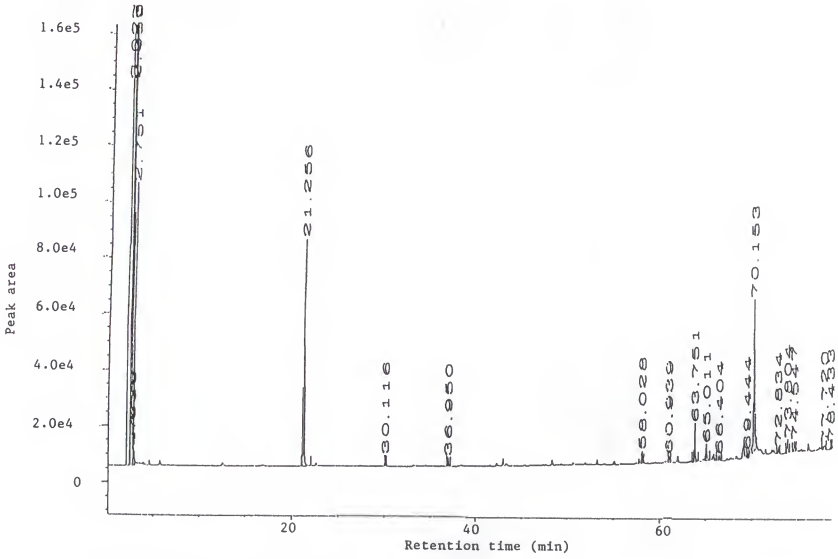


Figure C-1. Pollenkitt GC trace, pollen of Ilex vomitoria, spring 1995.

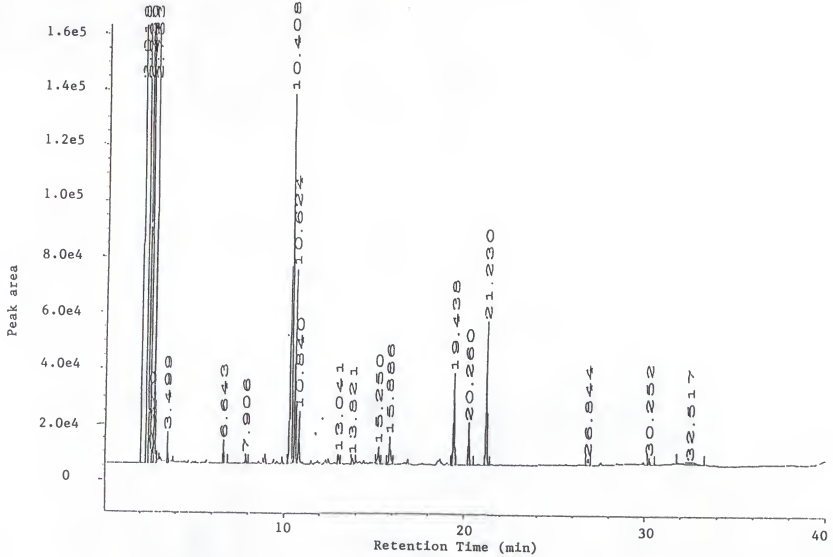


Figure C-1. Floral volatile GC trace, Ilex vomitoria, spring 1995.

APPENDIX D
CALCULATION OF SAMPLE CHEMICAL MASSES

The following is a sample calculation of the mass of a chemical emitted by a flowering male I. vomitoria in a one hour sample. The calculation uses data obtained by gas chromatographic analysis of a mixture of sample extract with internal standard so that each µl of the mixture contains 40 ng of the internal standard.

Source: A36
Date: March 19, 1995
Diel Period: 7

Data

Peak area α-pinene (A_{ap})= 16193
Peak area internal standard (A_{is})= 231660
Mean value for all internal standard peak areas (A_{Mis})= 201305

Calculation

1. Standardization of α-pinene peak area
   Standardization Factor (SF)= A_{is}/A_{Mis} = 231660/201305 = 1.1508
   Standardized α-pinene (A_{Sap})= SF*A_{ap} = 1.1508*16193 = 18635

2. Mass calculation of standardized α-pinene peak area
   M_{ap} = [A_{Sap}/A_{Mis}]*40ng = [18635/201305]*40ng = 3.7 ng

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BIOGRAPHICAL SKETCH

Robert Gordon Stewart was born in Winchester, Massachusetts, on June 7, 1949. He graduated from Reading Memorial High School in Reading, Massachusetts, in 1967 and began undergraduate study at Bowdoin College the same year. He received the A.B. degree with a major in biology in 1971.

Following graduation Mr. Stewart entered Peace Corps service as a secondary science teacher in Zaire. Between 1971 and 1982 he taught in Zaire for a total of nine years. While in Zaire he also worked as a trainer of new Peace Corps science teachers for five training programs.

From 1982 to 1986 Mr. Stewart taught biology at Our Lady of Nazareth Academy in Wakefield, Massachusetts. In 1986 he returned to Zaire under the joint auspices of the University of Lowell and the State University of New York at Stony Brook for one year of field research on the spatial and temporal distribution of Culicoides spp. in the Lomako Forest.

Five more years of teaching secondary biology included one year at the EdCo Alternative High School of Boston,

Massachusetts, and two years each at the Casablanca American School in Casablanca, Morocco, and the International School of Lusaka in Lusaka, Zambia. Mr. Stewart matriculated at the University of Florida in August 1992 to begin studies for attainment of the Doctor of Philosophy degree in entomology.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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